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Award Number: W81XWH-10-1-0777

TITLE: Repair of Nerve Cell Membrane Damage by Calcium-Dependent, Membrane-Binding Proteins

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REPORT DATE: September 2013

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
September 2013	Final	1 September 2010 - 31 August 2013

4. TITLE AND SUBTITLE
Repair of Nerve Cell Membrane Damage by Calcium-Dependent, Membrane-Binding Proteins

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0777

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Virginia
Charlottesville, VA 22903

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this study it was determined that human calcium-dependent, membrane-binding proteins of the annexin or copine classes are effective at preventing damage and repairing damage to lipid membranes caused by osmotic shock or other chemical and physical stresses using an in vitro assay involving synthetic membrane vesicles. These proteins may therefore be promising agents for protecting or promoting the repair of cell membranes in the case of spinal cord injury.

15. SUBJECT TERMS
Spinal cord injury, membrane repair

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	42	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

In acute spinal cord injury the plasma membranes of spinal neurons are torn allowing high concentrations of calcium to enter the cytoplasm, activating proteolytic cascades and leading to neuronal cell death. Membrane repair mechanisms have evolved that protect cells from this type of damage by repairing the cell membrane as soon as the increase in intracellular calcium is sensed by calcium-binding proteins. If these repair mechanisms can be strengthened either before or after spinal cord injury it may be possible to reduce cell damage resulting from the injury. In this project we are testing the hypothesis that the action of copine, a human calcium-dependent-membrane-binding protein, in model systems can promote a stable repair of broken membranes that could preserve cell viability. Preliminary data obtained using a novel imaging technology, atomic force microscopy, suggested that calcium-dependent, membrane-binding proteins of the copine class can repair membranes through direct binding to the edges of torn membranes and promoting sealing of the edges.

BODY OF REPORT

Research accomplishments associated with tasks (Aims) described in the statement of work.

Aim 1. Express human copine as a recombinant protein in yeast and purify.

Human copine I (a close homolog of copine VI expressed in neural tissue) was expressed from a galactose 10 (*GAL10)* -regulated yeast expression plasmid and purified by calcium-dependent binding to phospholipids followed by ion exchange chromatography (Figure 1). Yield was approximately 2 mg per four liter yeast culture. Human annexins I and VI, also calcium-dependent, membrane-binding proteins, were similarly expressed and purified from yeast for comparison with copine in the experiments described below.

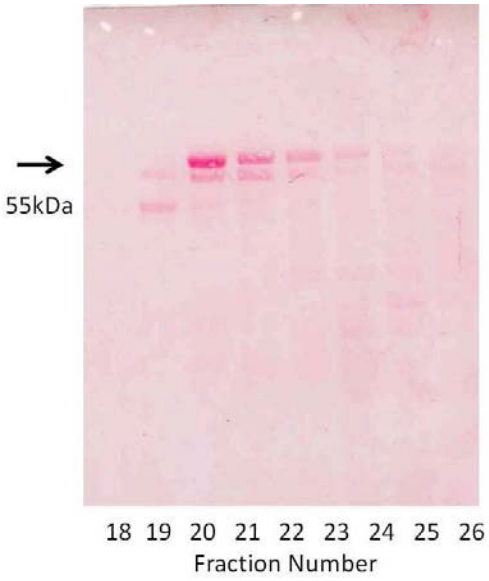

Figure 1: Electrophoretic analysis of purified human copine. Fractions 18 through 26 from the final purification step on a Fast Protein Liquid Chromatography (FPLC) system using the anion exchanger Poros Q were applied to an Sodium Dodecyl Sulfate (SDS) gel. After electrophoretic speparation the gel was blotted to nitrocellulose and the proteins stained with Ponceau S. The prominent band at 55 kilodalton (kDa) is copine; the shadow band slightly lower is believed to be a proteolytic breakdown product of copine.

Aim 2. Examine the association of copine with supported lipid bilayers containing defects by atomic force microscopy (AFM) to assess the ability of the protein to repair the bilayer defects.

A number of methods were tested to reliably prepare damaged membranes as a substrate for these experiments, including mechanical scoring of a supported bilayer with the AFM probe. The most reliable method was found to be limiting the amount of lipid applied to the substrate and reducing the time of incubation after application of the lipid to the substrate.

Clear documentation was obtained that the protein associated with the regions of membrane defect, and, over time tended to repair the defect so that a continuous bilayer was obtained (Figures 2 and 3). However, regions of the disrupted bilayer that had not been extensively imaged with the AFM probe did not exhibit the same extent of repair. This made it apparent that the mechanical action of the probe on the lipid bilayer was contributing to the repair, probably by transporting some lipid into the damaged areas of the membrane. Indeed, very forceful application of the probe was able to repair membranes alone without the addition of copine (Figure 4). Since the AFM could not be used in this fashion as a practical repair tool for damaged spinal cord membranes it became important to establish the assay using lipid vesicles described below (Aim 4) to test the functionality of the protein in membrane repair.

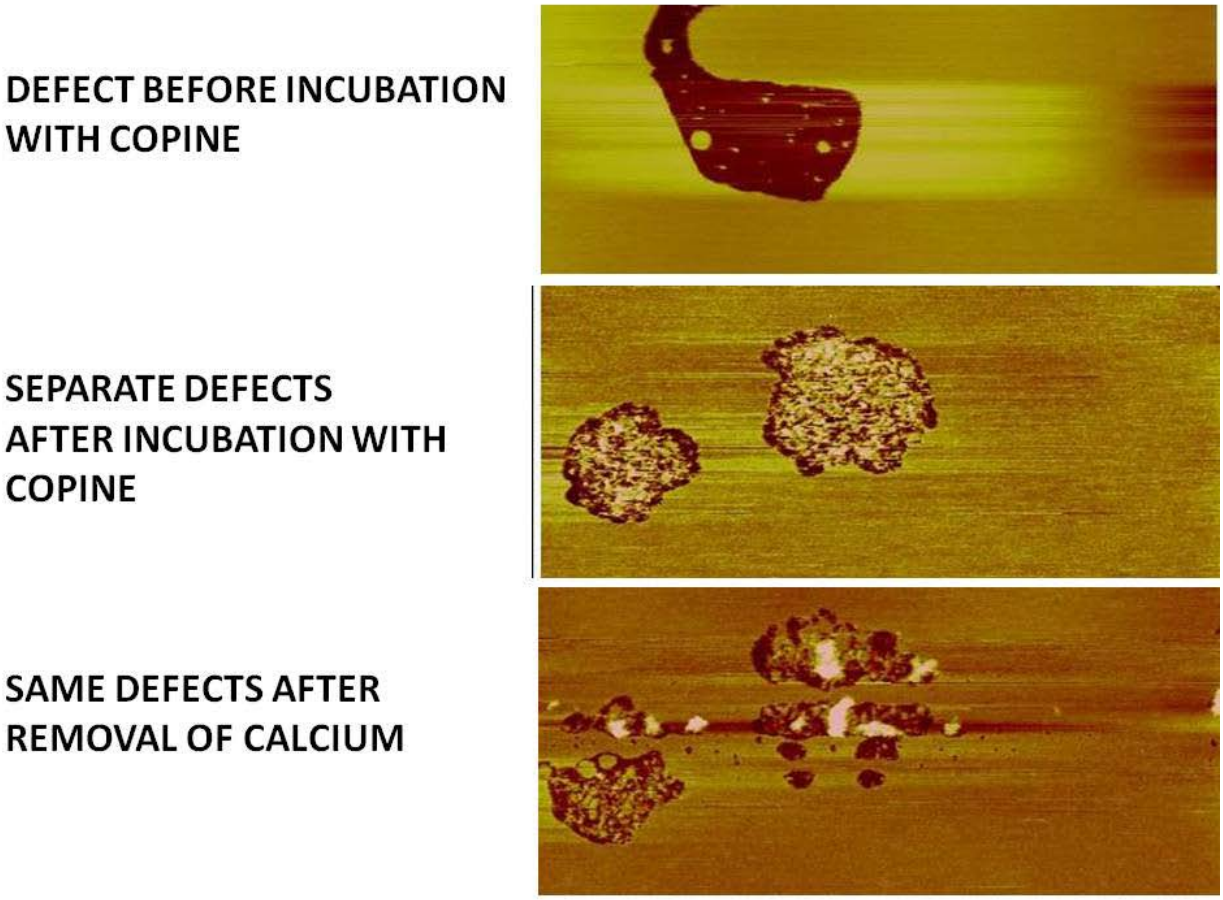

Figure 2: Application of copine to a supported bilayer with defects results in attachment of copine to the defects and formation of a continuous bilayer at the site of the defects.
Top: DEFECT BEFORE INCUBATION WITH COPINE. By limiting the amount of lipid applied to the mica substrate, and the time of incubation, it was found that bilayers with holes (dark areas) in them could be reproducibly formed. Visualization by atomic force microscopy, width of field 3micrometers.

Middle: SEPARATE DEFECTS AFTER INCUBATION WITH COPINE. In a different field of view on the same membrane after incubation with copine for 40 minutes, similar defects are partially filled with material representing both copine and transported lipid.

Bottom: SAME DEFECTS AFTER REMOVAL OF CALCIUM. In order to visualize the membrane underneath the accumulated copine protein, the membrane was incubated in the calcium chelator ethylene glycol tetraacetic acid (EGTA). In the absence of calcium copine is released from the membrane and reveals portions of continuous bilayer partially filling the original holes. The white material is aggregated protein and/or lipid.

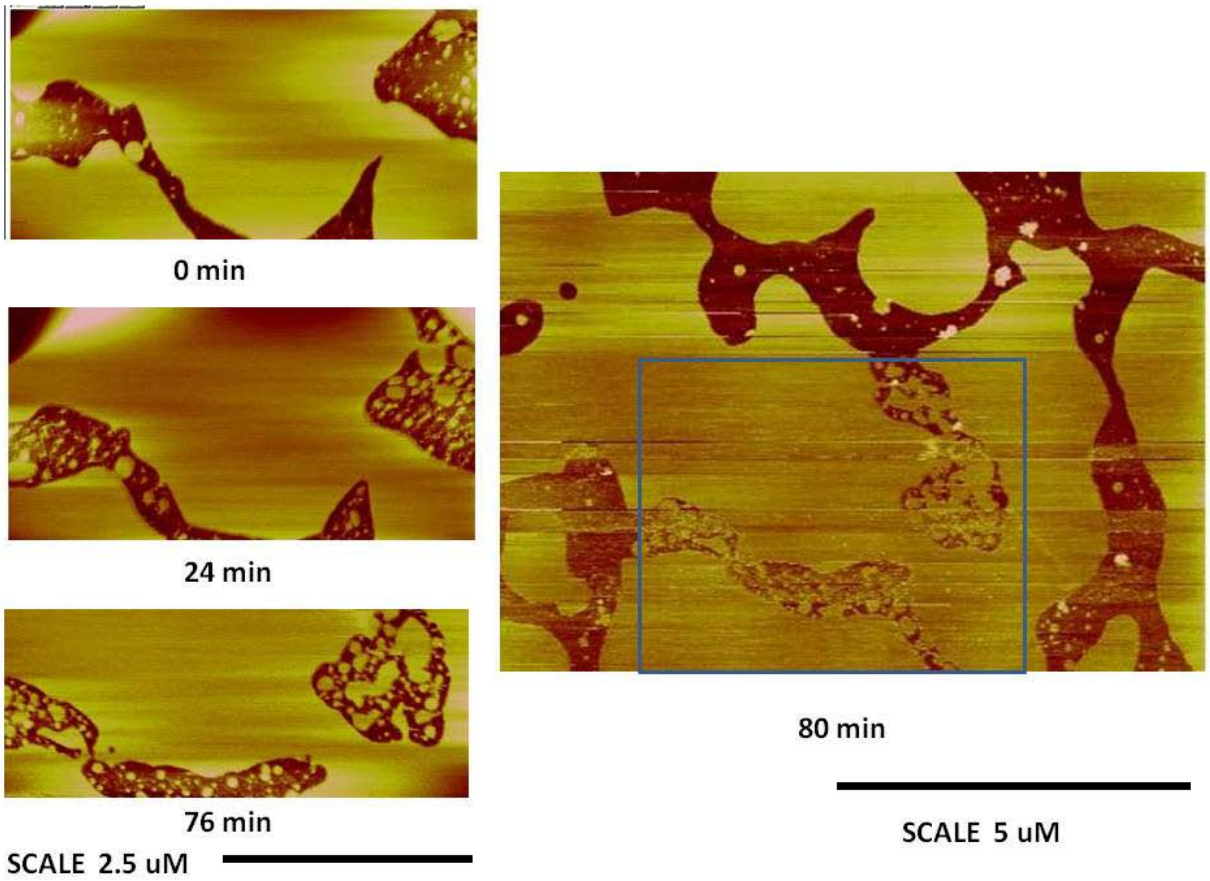

Figure 3: Bilayer repair is accelerated by the addition of lipid vesicles and is dependent on action of the AFM probe. ***0 min****: A bilayer is initially formed with significant defects as in Figure 2.* ***24 min, 76 min:*** *After addition of copine and brain lipid vesicles (Folch fraction I) and incubation for 24 or 76 minutes, new patches of bilayer appear in the membrane holes and defects.* ***80 min:****At 80 minutes after addition of copine and lipid vesicles the field of view is expanded to 10 by 10 micrometers revealing that membrane deposition and repair was dependent on the action of the AFM probe during the imaging (area inside the blue box corresponding to the areas imaged in the left panels).*

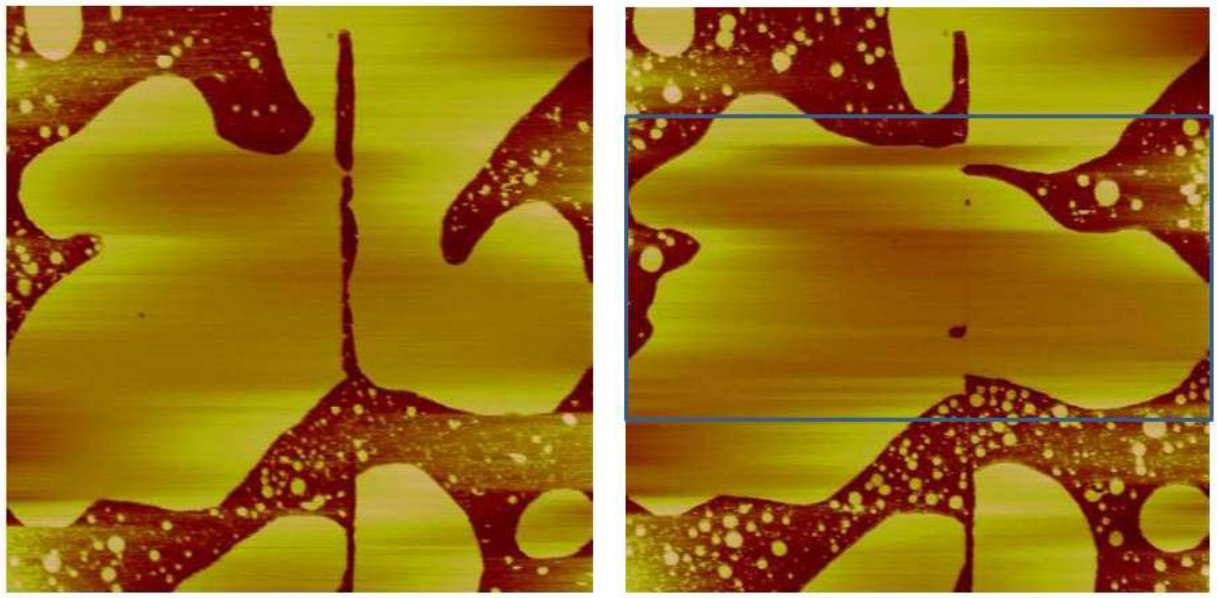

Figure 4:The action of the AFM probe alone can repair broken membranes. ***BEFORE:*** *An incomplete supported bilayer was further damaged by scoring it with the AFM probe in contact mode with high force. This resulted in the formation of the dark vertical wound crossing the bilayer patches. This wound is stable to imaging when the AFM is operated in tapping mode with normal imaging force (i.e., the region on the left was continuously imaged with several passes of the probe and the membrane topography did not change).* ***AFTER:*** *The same area was then subjected to increased force of the tapping probe (too great to permit imaging) in the area outlined by the blue box. In subsequent examination with a normal imaging force, as shown on the right, it is apparent that the vertical wound has been repaired by the action of the probe.*

Aim 3. Determine whether the addition of lipid vesicles would enhance the repair process mediated by copine in supported bilayers.

Evidence was obtained that the addition of vesicles to the medium over the bilayer resulted in greater transport of lipid into the regions of broken membrane. This is also shown in Figure 3 above. However, as in the case described above without vesicles, it was apparent that the AFM probe was contributing significantly to the repair process.

Aim4. In order to verify that true membrane sealing has been promoted by copine, a complementary assay will be developed based on a measure of the ability of the protein to reseal large unilamellar vesicles after rupture of the vesicles by osmotic shock.

Studies conducted under this Aim resulted in the establishment of liposome model for membrane damage by osmotic shock or other disruptive agents. In using the model to compare copine with another class of membrane-binding proteins, the annexins, we found that the annexins were much more effective at repairing membranes than copine, and therefore we focused our efforts on the annexins. These results are summarized in the sections below.

Methods used to establish a liposome model for membrane damage and repair

Unilamellar CF-loaded liposomes were prepared by extrusion through 100 nm Nuclepore polycarbonate filters using an extruder from Avanti Polar Lipids [1,2]. The lipids were mixed in appropriate ratios from stocks in chloroform, dried under a stream of argon and overlaid with a solution of 100 mM CF adjusted to pH 7.4 with NaOH. After extrusion the liposomes were separated from free dye on a Sephadex G-25 column equilibrated in 100 mM KCL, 50 mM HEPES-NaOH, pH 7.4.

Measurement of the dequenching of CF upon release from the liposomes as initially described by Weinstein and colleagues [3,4] was performed in a SPEX Fluorolog 111c spectrofluorometer with excitation at 495 nm and emission at 525 nm. Samples were incubated in a 5 by 5 mm quartz cuvette in a volume of 300 µl at 37 degrees C in 100 mM KCl, 50 mM HEPES-NaOH pH 7.4, 1 mM $CaCl_2$ ("Assay Buffer"). Fluorescence intensity was recorded as counts per second (cps) which represents actual photomultiplier counts per second divided by the value of the current from the reference detector (0.01 µA). Experiments were initiated by adding 10 µl of a liposome suspension containing 1 mg per ml of lipids to the final volume of 300 µl (final lipid concentration 33 µg/ml). Total releasable CF was determined after experiments by adding 10 µl 10% Triton X-100 and typically produced a fluorescence intensity of 0.5 to 1.0 X 10^8 cps. The initial percentage of free CF in the vesicle preparations was typically 5% to 10% of the total CF and experiments were designed so that not more than 50% of the CF was released during the time course of observation. For critical titrations and the osmotic shock experiments the Triton X-100 intensity values were used to normalize the data.

For the osmotic shock experiments 10 µl of the 1mg/ml vesicle suspension was incubated in the bottom of the fluorometer cuvette with 5 ul 100mM KCl 25 mM HEPES-NaOH, 1mM EGTA (FPLC fraction buffer) containing various amounts of annexin protein and 3 µl of 7.5 mM $CaCl_2$ (final free Ca^{2+} concentration 1.0 mM) and then diluted with Assay Buffer containing 3.5 or 5 mM $MgCl_2$ or the same buffer without KCl to remove osmotic support.

Measurements of the turbidity of vesicle suspensions were performed at 350 nm in a Beckman DU7 recording spectrophotometer in a volume of 1 ml.

Recombinant human annexin A5[5] and A6[6] were prepared by expression in yeast and isolation by calcium-dependent binding to multilamellar liposomes prepared from bovine brain Folch Fraction I lipids (Sigma-Aldrich) and ion exchange chromatography on Poros Q medium using a Pharmacia FPLC system[5].

Strategy for establishing the membrane leakage model

The strategy employed in these studies was to encapsulate carboxyfluorescein (CF) in liposomes at a self-quenching concentration and to monitor leakage by continuous measurement of the fluorescence increase associated with CF leakage and dequenching. The liposomes were monitored for "baseline" leakage and were exposed to a number of agents expected to perturb the membrane permeability barrier. Annexins were added to the exterior medium in the presence of Ca (1 mM) to promote binding of the annexin to the liposome in order to assess the effects this had on membrane permeation of CF. Since CF is a large (molecular weight 376 Da), negatively charged compound it does not readily pass through the bilayer and the leakage reflects significant disruption to bilayer structure.

Most members of the annexin family exhibit a "bivalent" activity resulting in the aggregation of membranes coincident with the binding of the annexin to the membrane [7,8]. Such membrane aggregation could potentially make the leakage data more complicated to interpret. The free liposome surface area exposed to the external medium would be reduced and therefore an annexin that promoted

membrane aggregation might appear to reduce leakage for this reason. Alternatively, membrane-membrane aggregation might be associated with distortion of the liposome geometry introducing regions of high curvature that might be more permeable, thus leading to greater leakage. In addition, in the presence of some of the membrane perturbants used in this study, membrane-membrane aggregation caused by the annexin might be followed by membrane fusion [9] and such fusion may be associated with a transient increase in membrane permeability. For these reasons the studies described here focused on annexins A5 and A6 since they do not promote membrane aggregation. To confirm that these annexins do not aggregate membranes in the conditions of the experiments the turbidity of the vesicle suspensions (absorbance at 350nm) was measured and found to be stable during the time course of the experiments, except in certain cases as described in the sections below. In contrast, in control experiments with annexin A1, which does promote membrane aggregation, the turbidity of the vesicle suspension increased 3 to 4 fold during the same time period.

The liposomes used for these studies were prepared from a mixture of lipids in order to reflect the complexity of lipids in the cytoplasmic leaflet of the plasma membrane: PS, PC, PE, cholesterol in a ratio of 1:1:1:1 by weight (approximately 1:1:1:2 molar ratio). For each condition that was examined in the sections below, titrations of critical parameters were performed in order to establish conditions under which sustained leakage of CF could be observed, compatible with the time course of the hand-mixing experimental techniques. The concentration of Mg^{2+} was found to have a significant influence on the rate of CF leakage from these negatively charged liposomes and on the apparent ability of some of the agents used to permeabilize the membranes. Standard Mg^{2+} concentrations of 1, 3.5, and 5 mM were tested. For different perturbing agents a single Mg^{2+} concentration was typically selected that provided a significant, sustainable leak during the course of the experiments. Calcium was tested at the single concentration of 1 mM, reflecting the high levels of calcium that might be anticipated at a site of damage at the plasma membrane of a cell (or the membrane of a calcium-containing organelle). In some cases high concentrations of the added permeabilized agents promoted vesicle aggregation, therefore the leakage measurements were limited to lower concentrations of the agents at which this was not detected in turbidity measurements. Some poorly soluble agents were added in ethanol or DMSO as solvent. The amounts of these solvents were kept to a minimum to reduce leakage due to the solvent. Leakage rates due to the solvent are reported in the experiments below if they were above baseline leakage in the absence of solvent.

Effects of annexins on baseline leakage of CF from liposomes

When incubated at 37 degrees C in 100 mM KCl, 50 mM HEPES-NaOH pH 7.4, 1 mM $CaCl_2$ ("Assay Buffer"), the liposomes exhibited a "baseline" leakage of CF that was enhanced by increasing the concentration of Mg^{2+} (Figure 5). When the liposomes were added to the cuvette with the annexin already present in solution in the cuvette the slopes of the CF release curves were analyzed after one to two min (Figure 5A). At 1 mM Mg^{2+} leakage was low and only slightly affected by the binding of either annexin which caused a 0 to 10% decrease in slope during the time course of the experiment (up to 20 min). At 3.5 mM Mg, binding of annexin A5 resulted in a very significant 43% reduction in leakage (Figure 5A) and annexin A6 a more modest 12% reduction in leakage. At 5 mM Mg^{2+}annexin A5 caused a 42% reduction in slope; the effect of annexin A6 was not determined at this Mg^{2+} concentration. When the liposomes were preincubated in the cuvette first and then the annexin was added it was possible to observe an initial burst of release of CF apparently caused by the initial binding of the annexin to the liposome membrane (Figure 5B). Subsequent to this event, the rate of release was reduced by the presence of the annexin. In the experiment illustrated in Figure 5B annexin A5 reduced the rate of release 11% at 1 mM Mg, 65% at 3.5 mM Mg, and 84% at 5 mM Mg. The size of the initial burst of CF release caused by the binding of the annexin, as seen in the figure, was larger in the presence of higher Mg^{2+} concentrations: This burst of CF release in 3.5 mM Mg^{2+} was 8 times higher than at 1 mM Mg^{2+}, and 12 times higher at 5 mM Mg^{2+}than at 1 mM Mg^{2+}.

When liposomes were added to Assay Buffer containing very high concentrations of divalent cations (10 mM Ca and 10 mM Mg^{2+} together) the liposomes rapidly aggregated giving a large increase in turbidity (absorbance at 350 nm) of 300% in 6 min and the CF was completely released during the first minute. At 1 mM Mg, 1 mM Ca no turbidity increase was seen. However, at both 3.5 and 5 mM Mg^{2+} in the presence of 1 mM Ca a small increase in turbidity was detectable (initial rate .026% per sec, compared to 10% per second in the presence of 10 mM Ca, 10 mM Mg) suggesting a slow process of vesicle aggregation may have been occurring, although other changes in membrane organization such as divalent cation-induced lipid phase separation may also have contributed to this small increase in turbidity. To determine whether the increased rate of baseline CF efflux at the higher Mg^{2+} concentrations was dependent upon vesicle-vesicle aggregation, the release rate was analyzed as a function of the vesicle concentration, anticipating that the rate would be second order relative to vesicle concentration if vesicle-vesicle interaction was required to promote the release of CF. However, with 5 mM Mg^{2+}, 1 mM Ca in the buffer, doubling the vesicle concentration resulted in an increase in the rate of release of a factor of 1.91+/- 0.15 (average of two independent experiments) suggesting a first order dependence on the vesicle concentration. The higher Mg^{2+} concentrations (3.5 and 5 mM) therefore appeared to be directly influencing the intrinsic permeability of the liposome membranes and the protective effect of the annexins was evidently not due to suppression of vesicle aggregation.

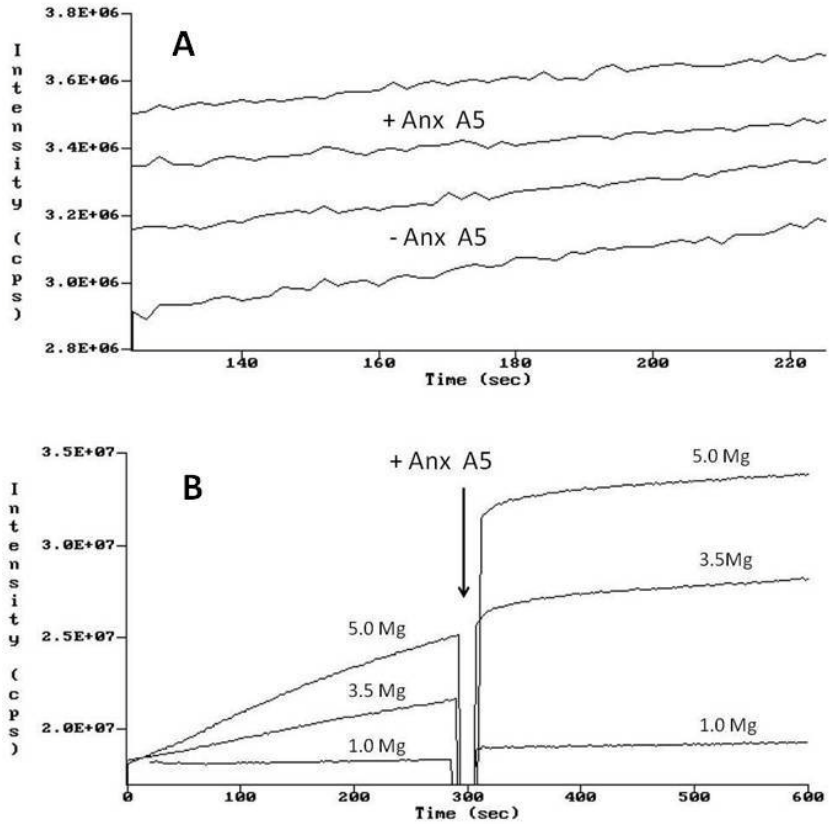

Figure 5: Reduction of baseline leakage of CF from liposomes by annexin A5. ***Part A****: Duplicate samples of liposomes (33µg lipid/ml) in the presence or absence of 4.8 µg/ml annexin A5 were incubated in Assay Buffer with 3.5 mM $MgCl_2$. Fluorescence intensity (cps – photomultiplier counts per second) is plotted as a function of time. Leakage (slope of the fluorescence trace) from samples without annexin is 2480 +/- 110 cps/sec, and with annexin A5 is 1420 +/- 160 cps/sec (a 43% reduction in slope due to the annexin). The individual traces have been translated along the vertical axis to separate them for clarity. Maximum fluorescence after adding Triton X-100 was 7.41 X 10^7 cps.* ***Part B****: Addition of annexin A5 to liposomes undergoing baseline leakage causes a burst of CF release after which the leakage rate is*

reduced. Liposomes were incubated in Assay Buffer with 1, 3.5, or 5 mM $MgCl_2$ as indicated. The annexin (4.50 µg/ml) was added at the arrow, at which point the fluorescence signal is lost during closure of the fluorometer shutters. Maximum fluorescence after adding Triton X-100 was 1.01 X 10^8 cps.

Effects of annexins on disruption of the membrane permeability barrier by arachidonic acid

Arachidonic acid is an important precursor for lipidic signaling molecules and is liberated by phospholipases from membrane phospholipids. As seen in Figure 6 when free arachidonic acid was added in ethanol as a vehicle it caused an increase in the liposome leakage rate (3.5 fold increase in slope). An initial burst of release of CF occurred when the arachidonate was added, but a burst of similar magnitude also occurred with an ethanol control and so may be due to a transient action of ethanol on a small number of vesicles before it is diluted. After this initial burst there was no increase in leakage due to the ethanol alone above the initial baseline rate. When the ethanol/arachidonate stock mixture was diluted three fold with Assay Buffer immediately before adding the arachidonate, there was no increase in slope due to the arachidonate and the initial burst was also almost eliminated. Therefore, it appears that if the arachidonate is allowed to form micelles in buffer before addition to the liposomes its transfer to the liposome membrane may be blocked on the time scale of the experiment.

When the liposomes were pre-incubated with 4.8µg/ml annexin A5 the leakage rate due to the addition of arachidonate was strongly suppressed as shown in Figure 6A. Similarly, when the same amount of annexin A5 was added after the arachidonate, it caused an initial burst of release of CF then it strongly inhibited the leak reducing it to a rate similar to that seen before addition of the fatty acid (Figure 6B). Annexin A6, however, added before the arachidonate at a similar concentration (4.9 µg/ml) caused an enhancement of the leakage due to arachidonic acid by 24%. At a two-fold higher concentration of annexin A6 (9.8 µg/ml) this stimulating effect on leakage was lost, but no protection from the permeability loss due to arachidonate was observed with annexin A6 at any concentration of protein. If added after the arachidonate, 4.9 µg/ml annexin A6 also caused an enhancement of the CF release rate by 38%.

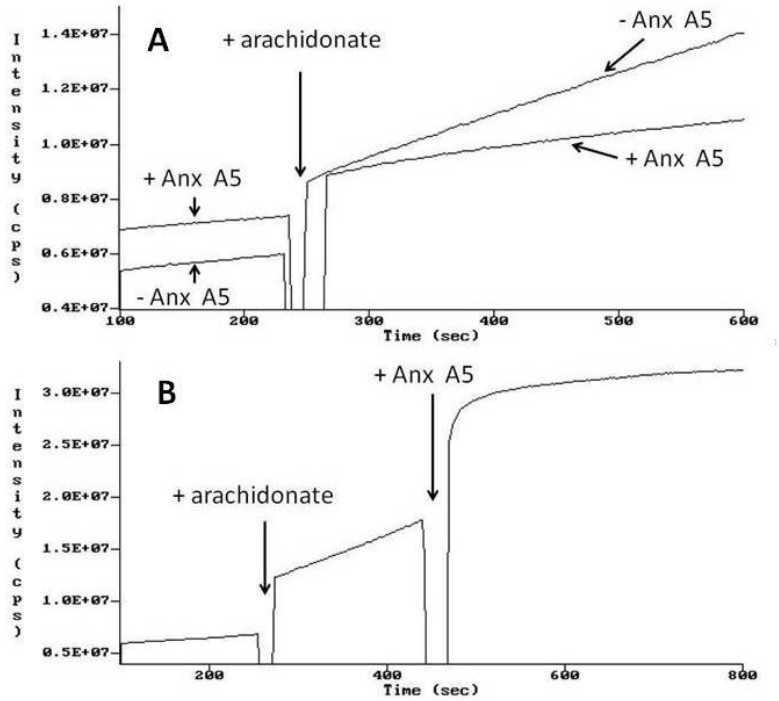

Figure 6: Annexin A5 protects membranes from leakage due to arachidonic acid. ***Part A****: Liposomes were preincubated in Assay Buffer with 5 mM $MgCl_2$ ("-* ***Anx A5****"). At 250 sec 2 µl of 0.4 mg/ml arachidonic acid dissolved in ethanol was added giving a final arachidonic acid concentration of 2.7 µg/ml ("+* ***arachidonate****"). This resulted in a burst of release of CF followed by continued leakage of CF at an increased rate as shown. When the liposomes were pre-incubated with 4.8 µg/ml A5 ("+* ***Anx A5****") the initial burst of CF release and the subsequent increase in release rate were inhibited as shown.* ***Part B****: Liposomes were pre-incubated in the absence of annexin A5. At 250 seconds 2.7 µg/ml arachidonic acid was added causing an increase in the rate of CF release. At 450 seconds 4.8 µg/ml annexin A5 was added which resulted in a burst of CF release followed by a suppression of the release rate to a level comparable to the initial rate.*

Effects of annexins on disruption of the membrane permeability barrier by lysophosphatidic acid (LPA)

Lysolipids such as lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA) are generated by the action of phospholipase A2 in the process of liberating arachidonic acid as a precursor to prostaglandin and leukotriene signaling molecules. Both agents have significant detergent-like properties. As illustrated in Figure 7A the addition of 1-palmitoyl LPA to liposomes promoted an initial burst of release of CF followed by a high rate of continued release. Similar to its action with arachidonic acid, annexin A5 was found to virtually completely protect the liposomes from this leakage, whether added before or after LPA (Figure 7A,B).

In contrast to the results with annexin A5, when annexin A6 under these conditions was bound to the liposomes it caused a 3.6 fold greater initial burst of CF release when the LPA was added, and a 2.8 fold elevated leakage rate subsequent to the initial burst. When the annexin A6 was added after the LPA it also caused a slight increase (1.2 fold) in the release rate.

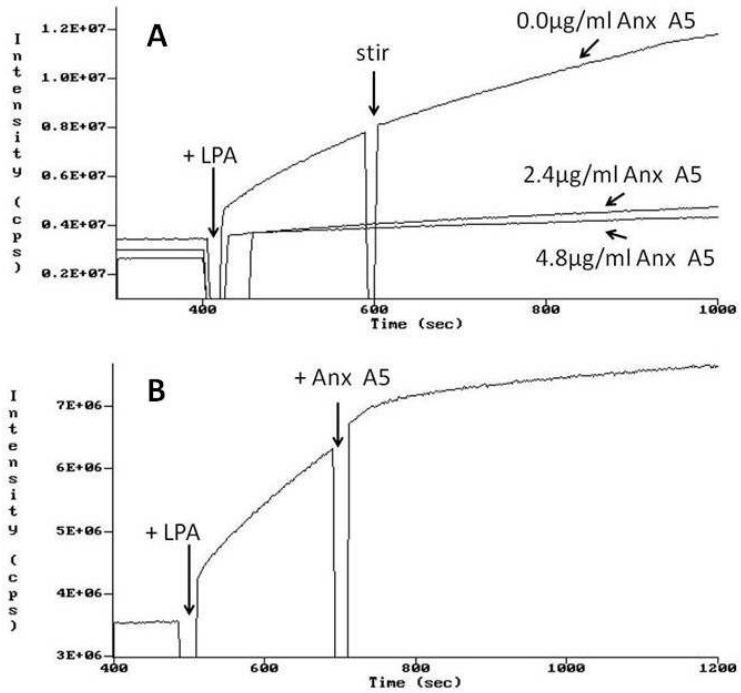

Figure 7: Annexin A5 inhibits the increase in CF leakage from liposomes due to addition of 1-palmitoyl lysophosphatidic acid (LPA). ***Part A****: LPA in 10 µl water was added to liposomes in Assay Buffer with 1 mM $MgCl_2$ at 450 seconds ("+LPA", final LPA concentration 6.7 µg/ml) causing an increase in the rate of CF release. When the liposomes were preincubated with 2.4 or 4.8 µg/ml annexin A5, as marked,*

*both the intial burst of CF release as well as the subsequent increase in release due to LPA were inhibited. At 600 seconds the LPA alone sample was stirred ("**stir**") as a control for the effects of stirring (compare with the annexin addition at 700 seconds in Part B). **Part B**: After addition of 6.7 µg/ml LPA at 500 seconds ("**+LPA**"), 4.8 µg/ml of annexin A5 was added at 700 seconds ("**+ Anx A5**") which reduced the rate of CF leakage.*

Effects of annexins on disruption of the membrane permeability barrier by lysophosphatidylcholine (LPC)

In contrast to LPA, LPC is zwitterionic and has a net neutral charge. It has been studied extensively for its effects on model membrane structure and has been attributed roles in membrane permeabilization and membrane fusion. In these experiments the addition of 1-oleoyl LPC in aqueous buffer or ethanol had only modest effects on liposome permeability at 1 mM Mg^{2+} concentration, acting to slightly enhance leakage (Figure 8A). At higher Mg^{2+} concentrations it appeared that larger amounts of LPC were able to interact with and/or enter the lipid bilayer. The initial interaction was associated with a large and rapid release of CF followed by stabilization of the membrane as CF permeability was then reduced below the original baseline (Figure 8A). This may have been due to a reduction of the surface charge of the bilayer as the neutral lipid was incorporated and to an alteration of lipid domain structure.

In order to establish a model for determining whether the annexins could protect against membrane permeabilization by LPC, attention was therefore focused on experiments with the lower concentration of Mg^{2+} (1 mM) in which the addition of LPC enhanced membrane leakage rather than reducing it. When pre-associated with the liposomes, both annexins A5 and A6 reduced the initial burst of CF release associated with mixing LPC with the liposomes, and caused a 44% (A5) or 38% (A6) reduction in the leakage rate (Figure 8B). If the annexins were added after the LPC, the leakage rate was reduced by 28% by annexin A5 and 51% by annexin A6.

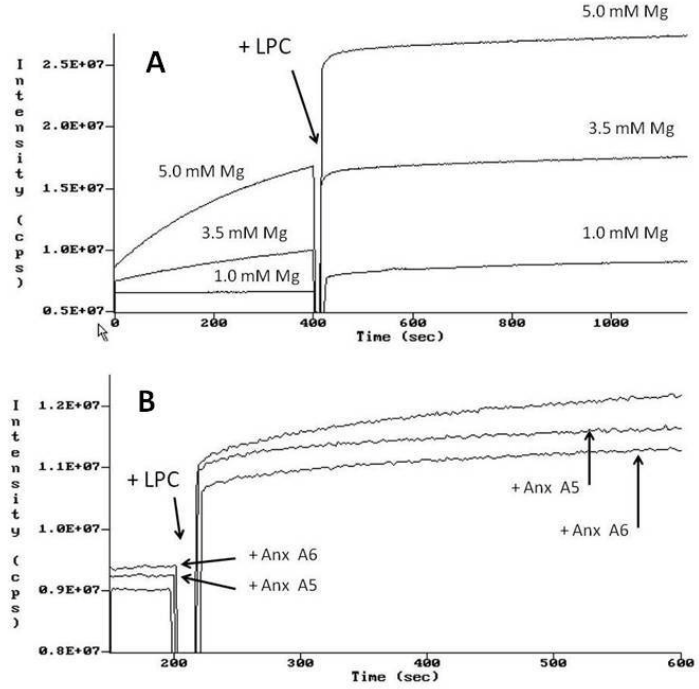

*Figure 8: Effects of 1-oleoyl lysophosphatidylcholine (LPC) and annexins on liposome permeability. **Part A**: LPC reduces liposome permeability at high magnesium concentrations. Liposomes were incubated in Assay Buffer with 1.0, 3.5, or 5.0 mM $MgCl_2$ as marked. At 400 sec LPC in 5 µl of water was added to give a final concentration of 16.7 µg/ml ("**+LPC**"). As shown, CF release was increased*

*by LPC at 1 mM $MgCl_2$ but reduced at the higher levels of $MgCl_2$. **Part B**: Annexins A5 and A6 reduce the leakage of CF from liposomes caused by the addition of LPC. Liposomes were incubated in Assay Buffer with 1 mM $MgCl_2$ in the absence of annexin or in the presence of 4.5 µg/ml annexin A5 ("**+Anx A5**") or 4.3 µg/ml annexin A6 ("**+Anx A6**"). At 200 sec LPC was added to a final concentration of 8.4 µg/ml ("**+LPC**").*

Effects of annexins on disruption of the membrane permeability barrier by diacylglycerol

Diacylglycerols are important intermediates in the biosynthesis and degradation of triglycerides, glycerophospholipids, and glyceroglycolipids and are known to act as second messengers in cell signaling through the activation of protein kinase C. Diacylglycerol has been found to cause alterations of membrane curvature, modification of surface charge, and promotion of bilayer to nonbilayer phase transitions. The addition of diacylglycerol (diolein) to the liposomes from an ethanol stock in the presence of 3.5 mM Mg^{2+} caused a significant, sustained leakage of CF (Figure 9). When annexin A5 was prebound to the liposomes it effectively blocked the increase in the release rate of CF due to the diacylglycerol after an initial burst of release that may have been due in part to the ethanol vehicle (Figure 9A). When the diacylglycerol was added first, the annexin A5 was effective in blocking the diacylglycerol-dependent leak, although the initial interaction of the annexin with the membrane caused a small burst of CF release before the release rate was returned to baseline levels (Figure 9B).

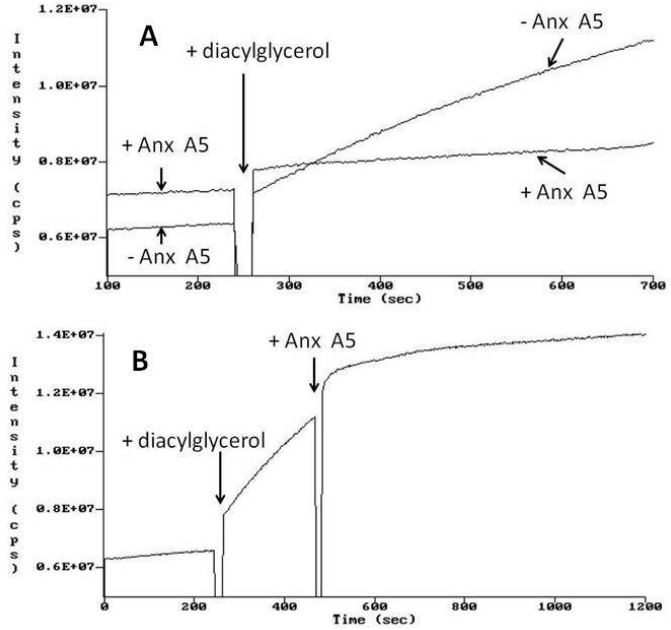

*Figure 9: Annexin A5 inhibits membrane leakage induced by diacylglycerol (diolein). Liposomes were incubated in Assay Buffer with 3.5 mM $MgCl_2$. **Part A**: At 250 sec diacylglycerol dissolved in 3µl of ethanol was added to give a final concentration of 1.0 µg/ml ("**+diacylglycerol**"). In one sample the vesicles were preincubated with 4.8 µg/ml of annexin A5 ("**+ Anx A5**") which blocked the increase in the rate of leakage after the initial burst of CF release that occurred when the diacylglycerol and ethanol were added. **Part B**: At 250 sec diacylglycerol was added as in part A to liposomes incubated in the absence of annexin. At 450 seconds annexin A5 was added to a final concentration of 4.5 µg/ml ("**+ Anx A5**"). After a burst of release of CF associated with the binding of the annexin to the liposomes, the subsequent rate of CF leakage was reduced to the baseline release rate in the absence of diacylglycerol.*

In contrast, annexin A6 at all levels tested caused an enhancement of the release due to diacylglycerol, although the effect was biphasic in that increasing the annexin A6 increased the release rate up to an

annexin concentration of 2.5 µg/ml, but a lower release rate was promoted by 4.9 µg/ml annexin (Figure 10).

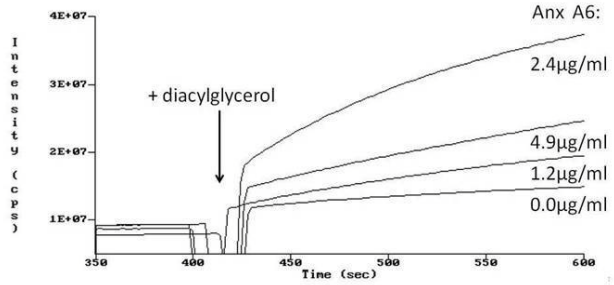

*Figure 10: Annexin A6 pre-bound to the liposomes enhances the leakage due to the addition of diacylglycerol (diolein) in a biphasic manner. Liposomes were pre-incubated with annexin A6 at the concentrations indicated. At 400 sec 3µl of diacylglycerol stock solution in ethanol was added to give a final diacylglycerol concentration of 1.0 µg/ml ("**+diacylglycerol**").*

Effects of annexins on disruption of the membrane permeability barrier by monoacylglycerol

Monoacylglycerols are generated through lipase action on triglycerides, and are also present as specific endocannabinoids such as 2-arachidonoylglycerol which function as signaling molecules. Similar to the affects of annexins on diacylglycerol treated liposomes, annexin A5 slightly inhibited CF leakage due to monoacylglycerol (monolein) and annexin A6 exacerbated the leakage due to monoacylglycerol (Figure 11).

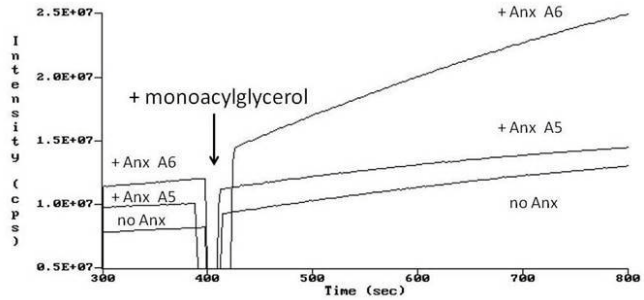

*Figure 11: Effects of annexins A5 and A6 on membrane permeabilization by monoacylglycerol. Liposomes were incubated in Assay Buffer with 3.5 mM $MgCl_2$. At 400 seconds monoacylglycerol (monolein) was added in 5µl of ethanol to a final concentration of 5.0 µg/ml ("**+monoacylglycerol**"). Preincubation of the liposomes with 4.5 µg/ml annexin A5 ("**+Anx A5**") reduced leakage, and with 4.3 µg/ml annexin A6 ("**+Anx A6**") increased leakage compared to monoacylglycerol alone ("**no Anx**").*

Effects of annexins on disruption of the membrane permeability barrier by spermidine.

The naturally occurring polyamines spermine, spermidine, and putrescine are polycations that are found at levels as high as millimolar in many cell types. Because of their cationic character polyamines bind to nucleic acids and also interact with anionic phospholipids in cell membranes. Some effects of polyamines on membrane properties have been described including membrane stabilization against osmotic stress, changes in membrane fluidity, changes in electrical conductivity, and effects on divalent cation-induced fusion of liposomes [10,11].

Annexin A5 pre-bound to the liposomes was highly effective at preventing leakage due to 2 mM spermidine in the presence of 1 mM Mg, reducing leakage to baseline levels at 4.5 µg/ml annexin and inhibiting leakage 50% at between 0.3 and 0.6 µg/ml (Figure 12A and Table 1). When annexin A5 was added after the spermidine it also suppressed leakage to baseline levels, but only after causing an abrupt and significant burst of CF release during the binding of the annexin to the liposomes (Figure 12B). This

initial burst of CF release due to the initial binding of the annexin was much greater than seen with liposomes in the absence of spermidine at 1 mM Mg^{2+} (compare with Figure 5B).

In similar experiments annexin A6 also reduced the leakage due to spermidine in the presence of 1 mM Mg, although it was less effective. If added before 2 mM spermidine at a concentration of 4.3 µg/ml it reduced the leak due to spermidine by 59.2% and if added after the spermidine at the same concentration it reduced the leak by 57.1%. The binding of annexin A6 to the liposomes did not cause the significant burst of release of CF as seen with annexin A5 in Figure 12B.

The addition of 2 mM spermidine under these conditions (1 mM Mg^{2+}) was found to cause a slow increase of the turbidity of the liposome suspension at a rate of .05% per sec relative to the initial turbidity of the vesicle suspension. This rate of turbidity increase was 200 fold less than the rate of turbidity increase seen when the vesicles were incubated in a mixture of 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (see section above on baseline release rates). This slow turbidity increase may have been due to vesicle aggregation caused by interaction of the positively charged spermidine with the negatively charged vesicles, or possibly reorganization of lipid domains in the membrane. In order to determine if vesicle aggregation may have contributed to the release of CF two independent experiments with different vesicle preparations were performed to determine the dependence of the rate of CF leakage upon vesicle concentration, similar to the strategy used to check for vesicle concentration effects on baseline leakage. Doubling the amount of vesicles in the assays resulted in a 2.08 +/- 0.61 fold increase in release rate of CF indicating a first order dependence of the release rate on vesicle concentration. This suggests the spermidine was acting directly to alter the intrinsic permeability of the membranes independent of vesicle aggregation and the protective effect of the annexins was therefore unlikely due to the suppression of vesicle aggregation.

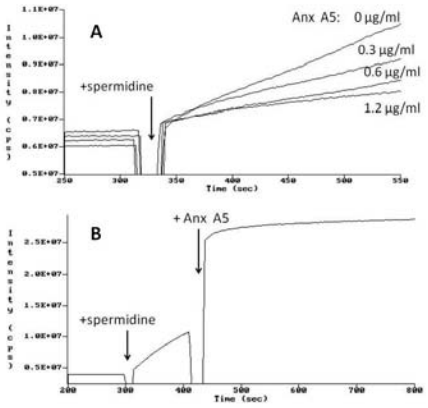

Figure 12: Annexin A5 suppresses CF leakage due to spermidine. ***Part A****: Liposomes were incubated in Assay Buffer with 1 mM $MgCl_2$ and with the concentrations of annexin A5 indicated. At 325 sec spermidine in water was added to a final concentration of 2 mM ("**+spermidine**"). Slopes of the traces after spermidine addition are given in Table 1.* ***Part B****: Addition of annexin A5 after spermidine caused a burst of CF release then stabilized the membrane leak. Spermidine was added at 300 sec to a final concentration of 2 mM ("**+spermidine**") . Annexin A5 was added at 425 sec to a final concentration of 1.9 µg/ml ("**+Anx A5**").*

Table 1: Effects of annexins A5 and A6 on the rates of release from liposomes treated with spermidine[a]

Annexin A5	Normalized Slope	Annexin A6	Normalized Slope
0.0 µg/ml	100	0.0 µg/ml	100
0.3 µg/ml	57.4	4.3 µg/ml	40.8

0.6 µg/ml	41.5		
1.2 µg/ml	28.4		
4.8 µg/ml	1.9		
0.0, µg/ml, no spermidine	1.5		

[a]*Spermidine concentration was 2 mM, other conditions as described under materials and methods and the legend to Figure 8. The slopes of the fluorescence versus time curves were normalized to the value in the presence of spermidine but absence of annexins.*

Effects of annexins on disruption of the membrane permeability barrier by amyloid-beta peptide 1-42 (A beta)

Alzheimer's disease is characterized by the buildup of fragments of the amyloid precursor protein, a neuronal plasma membrane protein, in extracellular spaces in the brain [12]. Soluble oligomers of the C-terminal fragment, amyloid-beta, or A-beta, have been demonstrated to be toxic to neurons so it has been hypothesized that A-beta contributes to the pathology of the disease [13]. A body of evidence suggests A-beta exerts its toxic effects through damaging the neuronal cell membrane and allowing excess calcium to enter nerve cells [13,14,15].
As shown in Figure 13, A-beta (12.8 µM) increased the release of CF from the liposomes and annexin A5 reduced this A-beta dependent leakage whether added to the liposomes before or after A-beta. The A-beta peptide was added to the vesicles in DMSO as a solvent. Control experiments indicated that DMSO alone had small but significant effects on the leakage rate and that this was suppressed by the annexin as well (Table 2). The effectiveness of the annexin had a very sharp dose-response titration providing no protection at 2.4 µg/ml and maximal protection at 3.6 µg/ml.

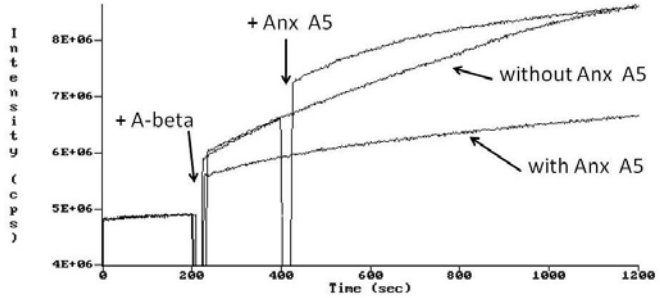

*Figure 13: Annexin A5 protects liposomes from permeabilization by A-beta. Liposomes were incubated in Assay Buffer with 1 mM $MgCl_2$. At 200 sec A-beta was added in 7µl DMSO ("**+ A-beta**") to a final peptide concentration of 12.8 µM and stimulated CF efflux (trace marked "**without Anx A5**"). When 4.8 µg/ml annexin A5 was incubated with the liposomes prior to addition of A-beta, the rate of CF release due to A-beta was reduced (trace marked "**with Anx A5**"). When the same amount of annexin was added after A-beta (trace following "**+ Anx A5**" at 400 sec) the rate of CF release was subsequently reduced to the rate seen when the protein was added before the A-beta peptide.*

Annexin A6 also inhibited the release of CF due to A-beta (5.5 µM), but with a very dramatic anomaly at an annexin concentration of 4.9 µg/ml at which the annexin enhanced release due to A-beta (Figure 14). Annexin A6 inhibited the slope due to A-beta by up to 50% before this anomaly; at the anomaly it caused a large burst of CF release followed by a leakage rate increased 13 fold above leakage due to A-beta alone (Table 2). At concentrations above this anomalous point annexin A6 again reduced the leakage due to A-beta by 50%. This anomalous effect of annexin A6 was seen if the annexin was added before or after A-beta (Figure 14B). Annexin A6 at this concentration (4.9 µg/ml) did not enhance the baseline release in the presence of the vehicle DMSO alone.

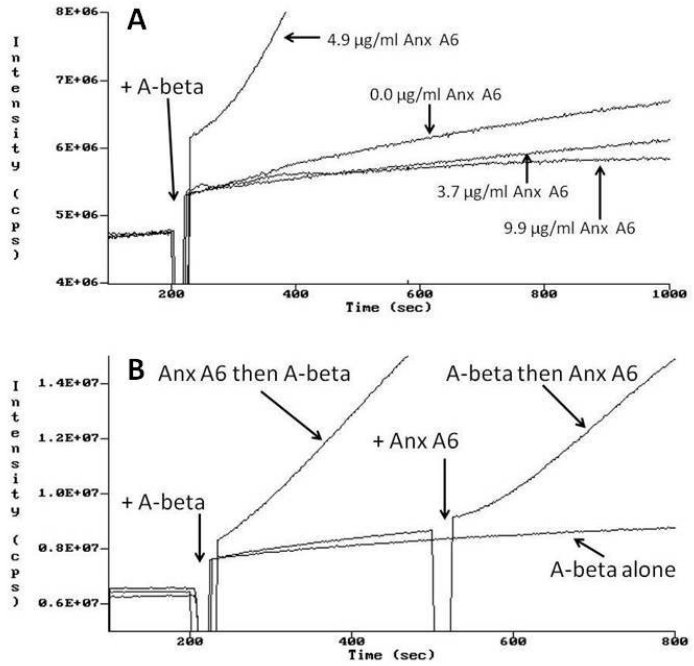

Figure 14: Effects of annexin A6 on CF leakage induced by A-beta. Liposomes incubated in Assay Buffer with 1 mM $MgCl_2$. ***Part A****: Liposomes were pre-incubated with 0, 3.7, 4.9, or 9.8 µg/ml annexin A6 and A-beta was added at 200 sec in 3µl of DMSO ("**+A-beta**") to a final peptide concentration of 5.5 µM. The top trace shows the enhanced leakage when the vesicles were incubated in 4.9 µg/ml annexin A6. The relative slopes of the traces are given in Table 2.* ***Part B****: Before 200 sec liposomes were preincubated in the absence (two samples) or presence (one sample) of 4.9 µg/ml annexin A6. At 200sec A-beta was added in 3 µl DMSO to give a final concentration of 5.5 µM peptide ("**+A-beta**") to all three samples. The sample containing annexin A6 exhibited a very high rate of release of CF ("**Anx A6 then A-beta**"). At 520 sec 4.9 µg/ml annexin A6 was added to one of the two samples without annexin that had A-beta previously added at 200 sec ("**A-beta then Anx A6**"). Note that the annexin strongly enhanced the leakage of CF induced by A-beta whether it was added before or after A beta.*

Table 2: Effects of annexins A5 and A6 on the rates of release of CF from liposomes treated with A-beta[a]

ANNEXIN A5		**ANNEXIN A6**	
Addition	Relative Slope	Addition	Relative Slope
None	100	None	100
DMSO	125	DMSO	157
A-beta	338	A-beta	504
A-beta + 1.2 µg/ml Annexin A5	345	A-beta + 1.2 µg/ml Annexin A6	357
A-beta + 2.4 µg/ml Annexin A5	375	A-beta + 2.5 µg/ml Annexin A6	264
A-beta + 3.6 µg/ml Annexin A5	108	A-beta + 3.7 µg/ml Annexin A6	407
A-beta + 4.8 µg/ml Annexin A5	62	A-beta + 4.9 µg/ml Annexin A6	6507
DMSO + 4.8 µg/ml Annexin A5	82	A-beta + 9.8 µg/ml Annexin A6	232

[a]A-beta was added at a final concentration of 5.5 µM from 3 ul of DMSO stock. DMSO controls were performed with 3 ul of DMSO alone. Slopes were normalized to 100 for the baseline slope in the absence of DMSO or A-beta. The slopes were determined 600 to 800 seconds after the addition of A-beta (corresponding to 800 to 1000 seconds in Figure 13). The experiments with annexin A5 and with annexin A6 were performed with different vesicle preparations. Other conditions were as described under Materials and Methods and in Figure 13 legend.

Effects of annexins on disruption of the membrane permeability barrier by amylin

In Type 2 diabetes there is a buildup of insoluble, fibrillar deposits of the peptide hormone amylin in the vicinity of the beta cells of the pancreas [16]. Soluble oligomers of amylin are apparently the precursors of these amylin fibrils and are in themselves toxic to the beta cells [17,18,19]. It has been hypothesized that these amylin oligomers contribute to the cause or severity of beta cell destruction in diabetes by a mechanism similar to that proposed for the action of A-beta on nerve cells, damaging the beta cell plasma membrane, allowing excess calcium to enter the cells.

When amylin was added to the liposomes it promoted the release of CF, and this release could be inhibited by annexin A5 whether the annexin was added to the liposomes before or after the addition of the peptide (Figure 15). At 4.8 µg/ml annexin A5 inhibited the enhancement of release by 6.4 µM amylin by 84%. When the same concentration of annexin A5 was added after the amylin (Figure 15B) the enhancement of release due to the amylin was reduced by 69%. Annexin A6 also provided protection of the membrane from permeabilization by amylin, but it was somewhat less effective than annexin A5: at 4.9 µg/ml annexin A6 the increase in release rate due to 6.4 µM amylin was reduced by 32%. However, annexin A6 did not promote an anomalous enhancement of CF release as was seen with A-beta even though used at similar concentration and conditions, including the use of DMSO as a vehicle.

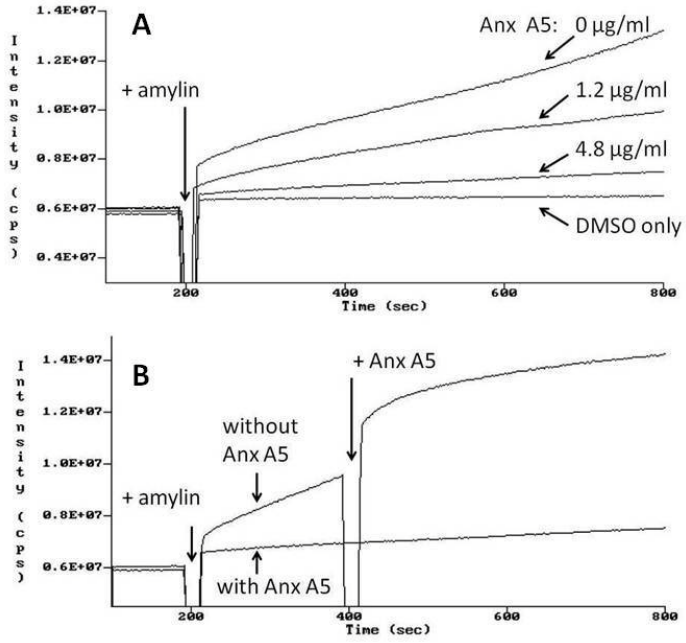

*Figure 15: Annexin A5 inhibits leakage of CF induced by amylin. Liposomes were incubated in Assay Buffer with 1 mM $MgCl_2$. **Part A**: Liposomes were pre-incubated with 0, 1.2, or 4.8 µg/ml annexin A5, as marked, and amylin was added at 200 sec in 3µl of DMSO to a final peptide concentration of 6.4 µM ("**+amylin**"). Trace marked "**DMSO only**": no annexin, 3 µl DMSO alone added at 200 sec. **Part B**: Liposomes were preincubated in the absence ("**without annexin**") or the presence ("**with annexin**") of 4.8 µg/ml annexin A5. At 200 sec Amylin was added in 3 µl DMSO to give a final concentration of 6.4*

*µM peptide ("**+amylin**"). At 400 sec 4.8 µg/ml annexin A5 was added to the sample initially without annexin.*

Effects of annexins on disruption of the membrane permeability barrier by osmotic shock

The annexins were tested for the ability to suppress leakage due to osmotic shock of liposomes, a type of stress that may serve as a model for membrane disruption due to mechanical means such as may occur in **muscular dystrophies, spinal cord injury, or traumatic brain injury**.

Liposomes were incubated in a small volume (15 µl) of buffer with 150 mM KCl for osmotic support (see Materials and Methods for details). After 200 seconds 290 µl of isotonic buffer (Assay Buffer containing 3.5 or 5 mM Mg) or hypotonic buffer (the same buffer without KCl) was used to dilute the sample approximately 20 fold in the fluorometer cuvette and the fluorescence was subsequently monitored continuously (Figure 16). When diluted with isotonic buffer there was no significant leakage of CF associated with the dilution and mixing. When diluted with hypotonic buffer there was a burst of release of approximately 17 % of the encapsulated CF. Subsequent to this burst there was an increase in the leakage rate relative to the control not exposed to the osmotic shock suggesting that although the membrane resealed after the shock, the permeability remained altered to some degree (Figure 16A). When annexin A5 was present in the initial incubation period (at an amount such that the final concentration after dilution was 4.5 µg/ml) the release of CF during hypotonic shock was reduced to 9% of the encapsulated CF, a reduction of 47% of the leakage during osmotic shock which was 17% (Figure 16A). Subsequent to the shock the continued presence of the annexin reduced the leakage of CF to the control level seen with unshocked liposomes (Figure 16A).

Experiments performed with annexin A6 (4.3 µg/ml after dilution) also showed protection from osmotic shock, although less than with annexin A5 (Table 3). The degree of protection with annexin A6 was greater when the experiment was performed with 5 mM Mg^{2+} than with 3.5 mM Mg^{2+}, while the degree of protection with annexin A5 was similar at the two Mg^{2+} concentrations (Table 3). Since the osmotic shock occurred at 200 seconds into the time course of the experiment, but mixing was not complete and monitoring of fluorescence did not occur until approximately 220 seconds, Table 3 also includes data obtained by extrapolating the lines in the graphs to 200 seconds.

The ability of annexin A5 to provide protection against osmotic shock was calcium-dependent. When the osmotic shock experiment was conducted in the presence of EGTA instead of 1 mM $CaCl_2$ the presence of annexin A5 had no effect on the release of CF due to mixing with hypotonic medium (Table 3). The protective action of annexin A5 occurred early in the process of osmotic shock. When the annexin was bound to the liposomes by the action of calcium in the pre-incubation step but the dilution was made with hypotonic buffer containing EGTA, which should promote the release of the annexin, suppression of the leakage due to the osmotic shock by 51% was observed (Figure 16B), although after the shock the rate of baseline release was not reduced by the annexin, as expected due to the removal of calcium (Figure 16 B). However, annexin A6 was not effective in this regard as it caused a slight increase in CF release that was not statistically significant upon osmotic shock with an EGTA containing buffer. The failure of annexin A6 to provide protection in this case may reflect a faster off rate for annexin A6 when the calcium was removed than occurs with annexin A5.

When the initial incubation was performed in 1 mM EGTA instead of 1 mM $CaCl_2$ and the dilution was performed in media containing 1 mM $CaCl_2$ there was no protection by annexin A5 from the osmotic shock, although the subsequent slow leakage was reduced by the annexin. Apparently the annexin was unable to move to the membrane quickly enough when the calcium-containing medium was added to provide protection from the initial impact of the osmotic shock.

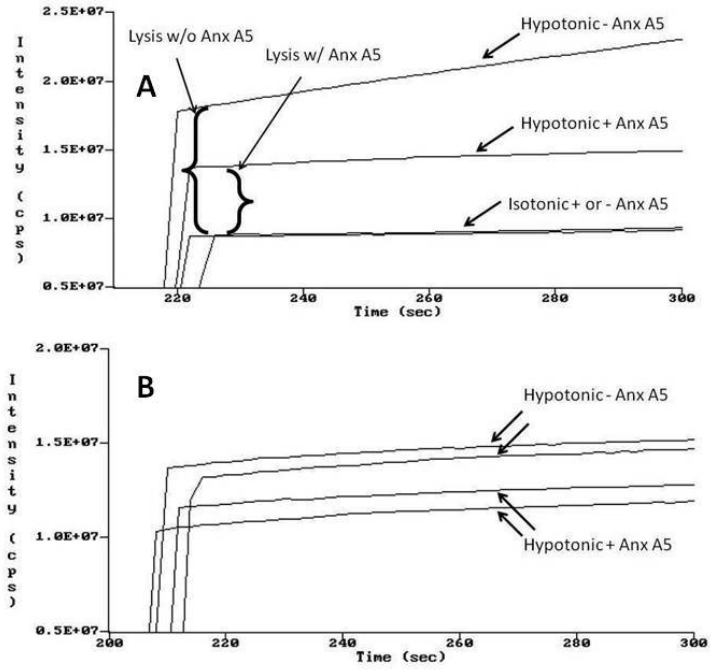

Figure 16: Annexin A5 suppresses the release of CF from liposomes exposed to osmotic shock. ***Part A****: Liposomes plus or minus annexin A5 were incubated in a small volume (15 µl) for 200 sec then were diluted to a volume of 305µl with isotonic or hypotonic buffer (see text for details). After the mixing was complete at approximately 220 sec the fluorometer shutters were opened and the fluoresence monitored continuously. Nearly coincident traces labeled "****Isotonic + or – Anx A5****" represent liposomes incubated with or without annexin A5 (4.5 µg/ml final concentration after dilution) and mixed with isotonic buffer. Trace "****Hypotonic - Anx A5****", lipsomes incubated without annexin A5 and mixed with hypotonic buffer; trace "****Hypotonic + Anx A5****", lipsomes incubated with annexin A5 and mixed with hypotonic buffer. The* ***brackets*** *mark the amount of CF released during the osmotic shock with or without annexin A5. Fluoresence intensity of suspensions after adding Triton was 6.7 X 10^7 cps.* ***Part B****: Annexin A5 provides protection against osmotic shock at an early time point. Liposomes were incubated in a small volume with or without annexin A5 and then diluted with hypotonic buffer containing 3.5 mM $MgCl_2$ and 2 mM EGTA in place of calcium. "****Hypotonic – Anx A5****", duplicate samples without annexin A5; "****Hypotonic + Anx A5****", duplicate samples with annexin A5. Fluorescence intensities at 220 sec: Without annexin 1.35+/-0.03 X 10^7 cps, with annexin 1.10+/-0.09 X 10^7 cps. Baseline intensity when diluted with isotonic buffer (not shown in the figure): 0.86+/-0.03 X 10^7 cps.*

Table 3: Effects of annexins A5 and A6 on the release of CF from liposomes subjected to osmotic shock

Annexin A5 or A6	**[Mg^{2+}]**	**% reduction in lysis[a]**	**% reduction extrapolated to 200 sec[b]**
A5	3.5 mM	46.9%	42.1%
A5	5.0 mM	47.3%	36.0%
A5 without Ca^{2+}	3.5 mM	0%	0%
A6	3.5 mM	16.5%	7.8%
A6	5.0 mM	28.6%	23.4%

[a]% reduction in lysis was determined by comparing the differences marked by the vertical brackets in the example in Figure 12. [b] % reduction extrapolated to 200 sec was determined by extending the

fluorescence traces, as seen in the example in Figure 12, to the time of initial mixing of the liposomes with the hypotonic medium.

Summary (Aim 4)

Annexin A5, and to a lesser extent annexin A6, was found in this study to stabilize the permeability barrier of complex liposomes against a wide variety of stresses, as summarized in Table 4.

Table 4: Summary of the effects of annexins A5 and A6 on the release of CF from liposomes subjected to stresses[a]

Agent or Action	Annexin A5	Annexin A6
None (Baseline)	Protection	Protection
Arachidonate	Protection	*Disruption*
Lysophosphatidic acid (LPA)	Protection	*Disruption*
Lysophosphatidylcholine (LPC)	Protection	Protection
Diacylglycerol	Protection	*Disruption*
Monoacylglycerol	Protection	*Disruption*
Spermidine	Protection	Protection
Amyloid-beta	Protection	Protection or *Disruption*[b]
Amylin	Protection	Protection
Osmotic Shock	Protection	Protection

[a]*The annexins either provided* ***Protection (****reduced the rate of release or CF) or caused* ***Disruption*** *(increased the rate of release of CF).*
[b]*Annexin A6 either provided protection or caused disruption in the presence of Amyloid-beta depending on the concentration of the annexin.*

Aim 5: In order to test whether copine can repair cell membranes in a living cell system the protein will be expressed in cultured neuronal cells. The cell membranes will be damaged by electroporation or mechanical injury and cell viability after injury compared to control cells not expressing copine.

As a prelude to these planned experiments we took advantage of the expression of copine and annexins in our yeast expression system and designed protocols for mechanical (sonication) or electroporation disruption of the yeast cell membranes. These preliminary studies did not, however, indicate that yeast cells expressing these proteins were more resistant to membrane damage than control yeast cells.

Expression systems for copine I and several annexins in neuronal cells were designed and constructed. However, funds for this project were exhausted before these expression systems could be tested in neuronal cells.

KEY RESEARCH ACCOMPLISHMENTS

1. Establishment of yeast expression systems for human copine and annexins.
2. Documentation of the ability of copine to repair supported lipid bilayers by atomic force microscopy.
3. Determination that the repair was influenced by the interaction with the atomic force microscope probe.
4. Establishment of an unsupported bilayer system consisting of liposomes for study of membrane repair.

5. Demonstration that annexins A5 and A6 promote repair of liposome membranes subjected to osmotic shock, excesses of amphiphilic molecules generated in metabolism or signaling, and cell damaging peptides involved in the pathogenesis of Alzheimer's disease and type 2 diabetes.

REPORTABLE OUTCOMES

1. U.S. Provisional Patent Application Serial No. 61/467,140
   Filed on March 24, 2011
   Title: Compositions and Methods for Maintaining and Repairing Membranes
   Reported to Edison on 3/8/11 under invention report #1526401-11-0016

2. Abstract presented at the Biophysical Society Meeting, San Diego, March 2012: C.E. Creutz, *Protection of the membrane permeability barrier by annexins.*

3. Publication: C.E. Creutz, J.K. Hira, V.E. Gee, and J.M. Eaton. Protection of the membrane permeability barrier by annexins. *Biochemistry* 51: 9966-9983 (2012).

PERSONNEL RECEIVING PAY FROM THE GRANT

Carl E. Creutz, Professor of Pharmacology
Virginia Erin Gee, Undergraduate Research Assistant

CONCLUSION

This project has demonstrated that calcium-dependent, membrane-binding proteins of the annexin and copine classes have the potential to directly repair or stabilize lipid bilayers. They may therefore be promising agents for repairing damaged cell membranes in the case of spinal cord injury.

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APPENDIX

Reprint of Publication: C.E. Creutz, J.K. Hira, V.E. Gee, and J.M. Eaton. Protection of the membrane permeability barrier by annexins. *Biochemistry* 51: 9966-9983 (2012).

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Protection of the Membrane Permeability Barrier by Annexins

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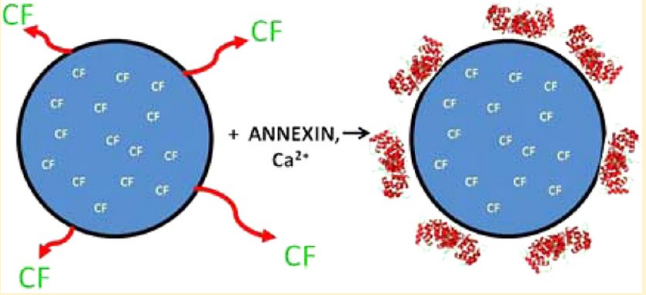
ABSTRACT: Biological membranes are exposed to a number of chemical and physical stresses that may alter the structure of the lipid bilayer in such a way that the permeability barrier to hydrophilic molecules and ions is degraded. These stresses include amphiphilic molecules involved in metabolism and signaling, highly charged polyamines, membrane-permeating peptides, and mechanical and osmotic stresses. As annexins are known to bind to lipid headgroups in the presence of calcium and increase the order of the bilayer lipids, this study addressed whether this activity of annexins provides a potential benefit to the membrane by protecting the bilayer against disruptions of this nature or can promote restoration of the permeability barrier after damage by such agents. The release of carboxyfluorescein from large unilamellar vesicles composed of lipids characteristically present in the inner leaflet of cell membranes (phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, and cholesterol) was used to measure membrane permeability. It was determined that in the presence of calcium, annexin A5 reduced the level of baseline leakage from vesicles and reduced or reversed damage due to arachidonic acid, lysophosphatidic acid, lysophosphatidylcholine, diacylglycerol, monoacylglycerol, spermidine, amyloid-β, amylin, and osmotic shock. Annexin A6 was also able to provide membrane protection in many but not all of these cases. In a cell, it is likely annexins would move to sites of breakdown of the permeability barrier because of the calcium-dependent promotion of the binding of annexins to membranes at sites of calcium entry. Because of the fundamental importance to life of maintaining the permeability barrier of the cell membrane, it is proposed here that this property of annexins may represent a critical, primordial activity that explains their great evolutionary conservation and abundant expression in most cells.



All life on Earth depends upon the properties of a remarkable structure only two molecules thick, the lipid bilayer that forms the hydrophobic permeability barrier of the cell membrane. During evolution, a number of mechanisms have evolved to protect the integrity of this structure. In eukaryotic cells, complex mechanisms involving cytosolic and membrane proteins that play roles in membrane trafficking events apparently function to repair outright ruptures of the plasma membrane.[1,2] The proteins involved include calcium-regulated proteins of the synaptotagmin[3] and annexin[4−8] classes that would be expected to be activated by calcium near sites of membrane disruption where the ion enters from the external milieu or internal stores and acts as a kind of "alarm signal" that membrane damage has occurred. However, more subtle and possibly more frequent damage to the permeability barrier of cell membranes may also occur because of the transient accumulation of amphiphilic molecules that can disrupt lipid bilayers by acting as detergents or by highly charged basic compounds like the polyamines that adhere strongly to acidic phospholipids. Such molecules are generated during the metabolism of lipids and other cell constituents and during the generation of signaling molecules. Examples of compounds that occur naturally and can alter bilayer structure are mono- and diacylglycerols, free fatty acids, lysophospholipids, and the polyamines spermine, spermidine, and putrescine. Membranes can also be damaged by mechanical stresses occurring during muscle cell contraction or extension and osmotic imbalances.

The annexins make up a highly conserved class of proteins found in plants and animals that bind to the surface of phospholipid bilayers in the presence of calcium.[4] Because annexins have a long evolutionary history, are abundant (up to several percent of the soluble proteins in cells), and are expressed from multiple related genes (12 gene products in humans[9]), it is perhaps not surprising that the annexins are now recognized to be involved in a number of unrelated processes. These include activities as diverse as regulation of the coagulation cascade in blood and the chaperoning of membrane interactions inside cells.[4] Common to these diverse functions is the ability to bind calcium and phospholipids, and the amino acid residues responsible for this regulated binding activity are the most highly conserved features of the annexins. This conservation substantiates the biological importance of their ability to bind lipids in a calcium-regulated fashion, but it raises the question of the primordial function of the annexins that depends on this binding activity and was sufficiently important to explain their conservation and radiation throughout the plant and animal kingdoms. We suggest here that the ability of

Received: October 4, 2012
Revised: November 27, 2012
Published: November 28, 2012

   dx.doi.org/10.1021/bi3013559 | *Biochemistry* 2012, 51, 9966−9983

annexins to maintain or repair the permeability barrier of membranes may be their most important function in evolutionary terms and that this activity depends upon their ability to bind to sites of membrane damage in a calcium-dependent manner.

The ability of annexins to modulate the permeability barrier of membranes has received only limited attention. Biophysical studies of annexin−membrane interaction have instead emphasized the ability of annexins to promote membrane−membrane contacts and to influence lipid organization into domains and the diffusion of lipids in the plane of the bilayer.[4,10−16] However, some annexins appear to bind preferentially to lipids that have a greater degree of disorder as they do not bind to gel phase lipids below the transition temperature in model membranes.[17] This may be a property that would enhance the association of these annexins with sites of membrane perturbation or disruption. Some studies have reported that annexins can influence the ability of channel-forming toxins or ionophores to promote conductances across bilayers.[18,19] This might translate into an ability of annexins to protect membranes from pore-forming toxins in vivo. On the other hand, some studies have indicated that annexins themselves can promote the permeation of ions across artificial membranes, possibly because of refolding and insertion of the annexin into the membrane at low pH[20] or electrostatic forces from the high dipole moment of the bound annexin promoting disruption of bilayer structure.[21,22] In this report, the effects of a number of natural amphiphiles or membrane disruptive agents on synthetic bilayers have been studied, and we found that annexins, acting alone in a calcium-regulated manner, have the ability to partially prevent or reverse the permeability changes caused by these agents.[a]

MATERIALS AND METHODS

Materials. Phospholipids and cholesterol for liposome preparation were from Avanti Polar Lipids: 1,2-dioleyl-*sn*-glycero-3-phosphocholine (PC), 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine (PE), L-α-phosphatidylserine from porcine brain (PS), cholesterol from ovine wool, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate (LPA), and 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC). 5,6-Carboxyfluorescein (CF) was obtained from Eastman, synthetic human amyloid-β 1−42 from Genscript or EZBiolab, and synthetic human amylin-amide from Genscript.

Methods. Unilamellar CF-loaded liposomes were prepared by extrusion through 100 nm Nuclepore polycarbonate filters using an extruder from Avanti Polar Lipids.[23,24] The lipids were mixed in appropriate ratios from stocks in chloroform, dried under a stream of argon, and overlaid with a solution of 100 mM CF adjusted to pH 7.4 with NaOH. After extrusion, the liposomes were separated from free dye on a Sephadex G-25 column equilibrated in 100 mM KCl and 50 mM HEPES-NaOH (pH 7.4).

Measurement of the dequenching of CF upon release from the liposomes as initially described by Weinstein and colleagues[25,26] was performed in a SPEX Fluorolog 111c spectrofluorometer with excitation at 495 nm and emission at 525 nm. Fluorescence kinetic data from the spectrofluorometer were captured with a model PDX-R Print Data Recorder from Photologic Inc. to generate the figures shown. Samples were incubated in a 5 mm × 5 mm quartz cuvette in a volume of 300 μL at 37 °C in 100 mM KCl, 50 mM HEPES-NaOH (pH 7.4), and 1 mM $CaCl_2$ (assay buffer). The fluorescence intensity was recorded as counts per second (cps), which represents actual photomultiplier counts per second divided by the value of the current from the reference detector (0.01 μA). Experiments were initiated by adding 10 μL of a liposome suspension containing 1 mg/mL lipids to the final volume of 300 μL (final lipid concentration of 33 μg/mL). The total amount of releasable CF was determined after experiments via addition of 10 μL of 10% Triton X-100 and typically produced a fluorescence intensity of 0.5−1.0 × 10^8 cps. The initial percentage of free CF in the vesicle preparations was typically 5−10% of the total amount of CF, and experiments were designed so that not more than 50% of the CF was released during the course of observation. For critical titrations and the osmotic shock experiments, the Triton X-100 intensity values were used to normalize the data.

For the osmotic shock experiments, 10 μL of the 1 mg/mL vesicle suspension was incubated in the bottom of the fluorometer cuvette with 5 μL of 100 mM KCl, 25 mM HEPES-NaOH (pH 7.4), and 1 mM EGTA (FPLC fraction buffer) containing various amounts of annexin protein and 3 μL of 7.5 mM $CaCl_2$ (final free Ca^{2+} concentration of 1.0 mM) and then diluted with assay buffer containing 3.5 or 5 mM $MgCl_2$ or the same buffer without KCl to remove osmotic support.

Measurements of the turbidity of vesicle suspensions were performed at 350 nm in a Beckman DU70 recording spectrophotometer in a volume of 1 mL.

Recombinant human annexin A5[27] and A6[28] were prepared by expression in yeast and isolated by calcium-dependent binding to multilamellar liposomes prepared from bovine brain Folch Fraction I lipids (Sigma-Aldrich) and ion exchange chromatography on Poros Q medium using a Pharmacia FPLC system.[27]

The figures presented are representative of data obtained in at least three independent experiments with qualitatively similar results. Parameters obtained from multiple determinations are presented as means ± sample standard deviations. Titration data (e.g., Tables 1 and 2) are derived from a single determination at each value of the independent variable.

RESULTS

Establishing a Membrane Leakage Model. The strategy employed in these studies was to encapsulate carboxyfluorescein (CF) in liposomes at a self-quenching concentration and to monitor leakage by continuous measurement of the fluorescence increase associated with CF leakage and dequenching.[25,26] The liposomes were monitored for "baseline" leakage and were exposed to a number of agents expected to perturb the membrane permeability barrier. Annexins were added to the exterior medium in the presence of Ca (1 mM) to promote binding of the annexin to the liposomes to assess the effects this had on membrane permeation of CF. Because CF is a large (molecular mass of 376 Da), negatively charged compound, it does not readily pass through the bilayer, and the leakage reflects a significant disruption of bilayer structure.

Most members of the annexin family exhibit a "bivalent" activity resulting in the aggregation of membranes coincident with the binding of the annexin to the membrane.[4,10] Such membrane aggregation could potentially make the leakage data more complicated to interpret. The free liposome surface area exposed to the external medium would be reduced, and therefore, an annexin that promoted membrane aggregation might appear to reduce the rate of leakage for this reason. Alternatively, membrane−membrane aggregation might be

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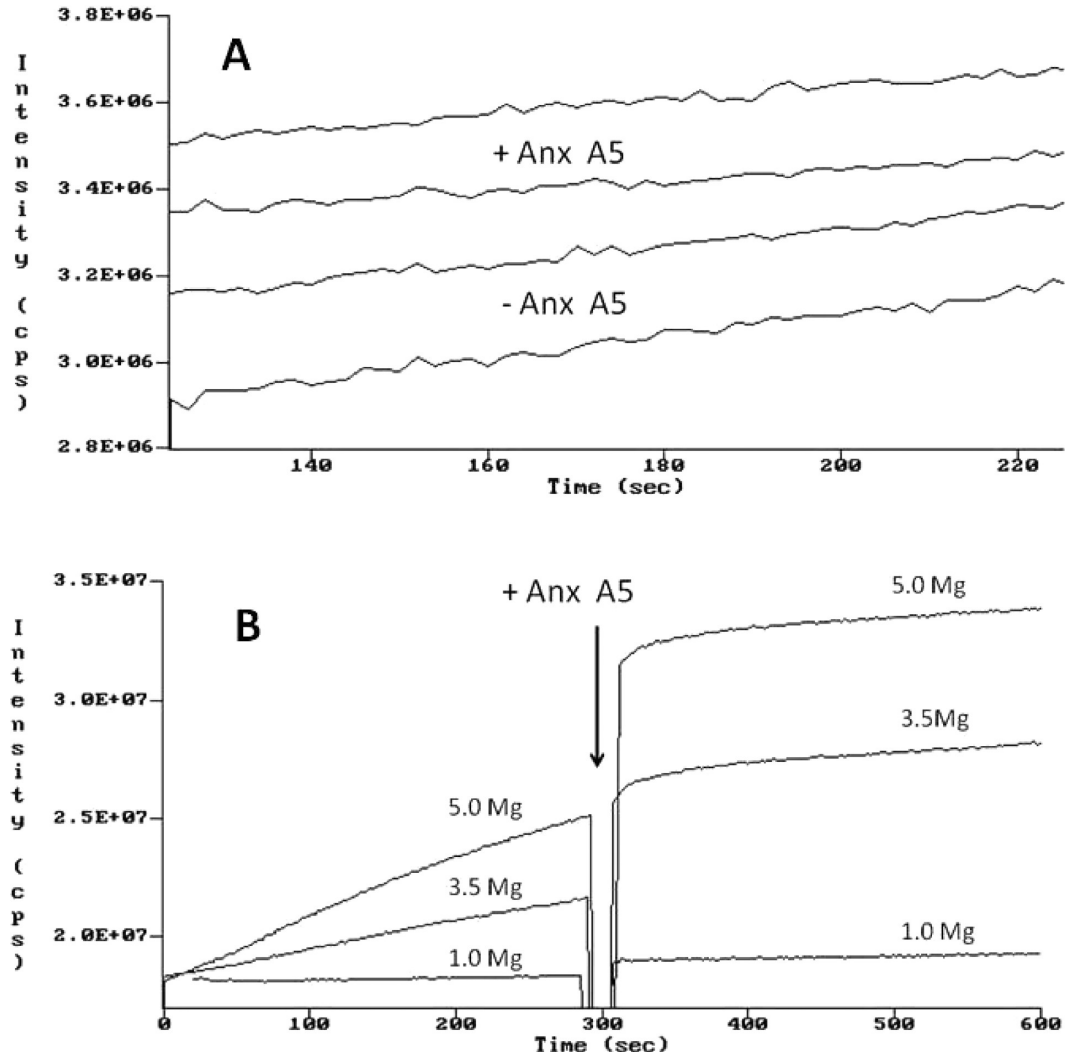

Figure 1. Reduction of the rate of baseline leakage of CF from liposomes by annexin A5. (A) Duplicate samples of liposomes (33 μg of lipid/mL) in the presence or absence of 4.8 μg/mL annexin A5 were incubated in assay buffer with 3.5 mM $MgCl_2$. The fluorescence intensity (cps, photomultiplier counts per second) is plotted as a function of time. The rate of leakage (slope of the fluorescence trace) from samples without annexin is 2480 ± 110 cps/s, and with annexin A5, it is 1420 ± 160 cps/s (a 43% reduction in slope caused by the annexin). The individual traces have been translated along the vertical axis to separate them for the sake of clarity. The maximal fluorescence after the addition of Triton X-100 was 7.41×10^7 cps. (B) Addition of annexin A5 to liposomes undergoing baseline leakage causes a burst of CF release after which the leakage rate is reduced. Liposomes were incubated in assay buffer with 1, 3.5, or 5 mM $MgCl_2$ as indicated. The annexin (4.50 μg/mL) was added at the arrow, at which point the fluorescence signal is lost during closure of the fluorometer shutters. The maximal fluorescence after the addition of Triton X-100 was 1.01×10^8 cps.

associated with distortion of the liposome geometry introducing regions of high curvature that might be more permeable, thus leading to more leakage. In addition, in the presence of some of the membrane perturbants used in this study, membrane−membrane aggregation caused by the annexin might be followed by membrane fusion,[29] and such fusion may be associated with a transient increase in membrane permeability. For these reasons, the studies described here focused on annexins A5 and A6 because they do not promote membrane aggregation. To confirm that these annexins do not promote membrane aggregation under the conditions of our experiments, we measured the turbidity of the vesicle suspensions (absorbance at 350 nm) and found it to be stable during the time course of the experiments, except in certain cases as described below. In contrast, in control experiments with annexin A1, which does promote membrane aggregation, the turbidity of the vesicle suspension increased 3−4-fold during the same time period.

The liposomes used for these studies were prepared from a mixture of lipids to reflect the complexity of lipids in the cytoplasmic leaflet of the plasma membrane: PS, PC, PE, and cholesterol in a ratio of 1:1:1:1 by weight (approximately 1:1:1:2 molar ratio). For each condition that was examined, titrations of critical parameters were performed to establish conditions under which sustained leakage of CF could be observed, compatible with the time course of the hand-mixing experimental techniques. The concentration of Mg^{2+} was found to have a significant influence on the rate of leakage of CF from these negatively charged liposomes and on the apparent ability of some of the agents used to permeabilize the membranes. Standard Mg^{2+} concentrations of 1, 3.5, and 5 mM were tested. For different perturbing agents, a single Mg^{2+} concentration was typically selected that provided a significant, sustainable

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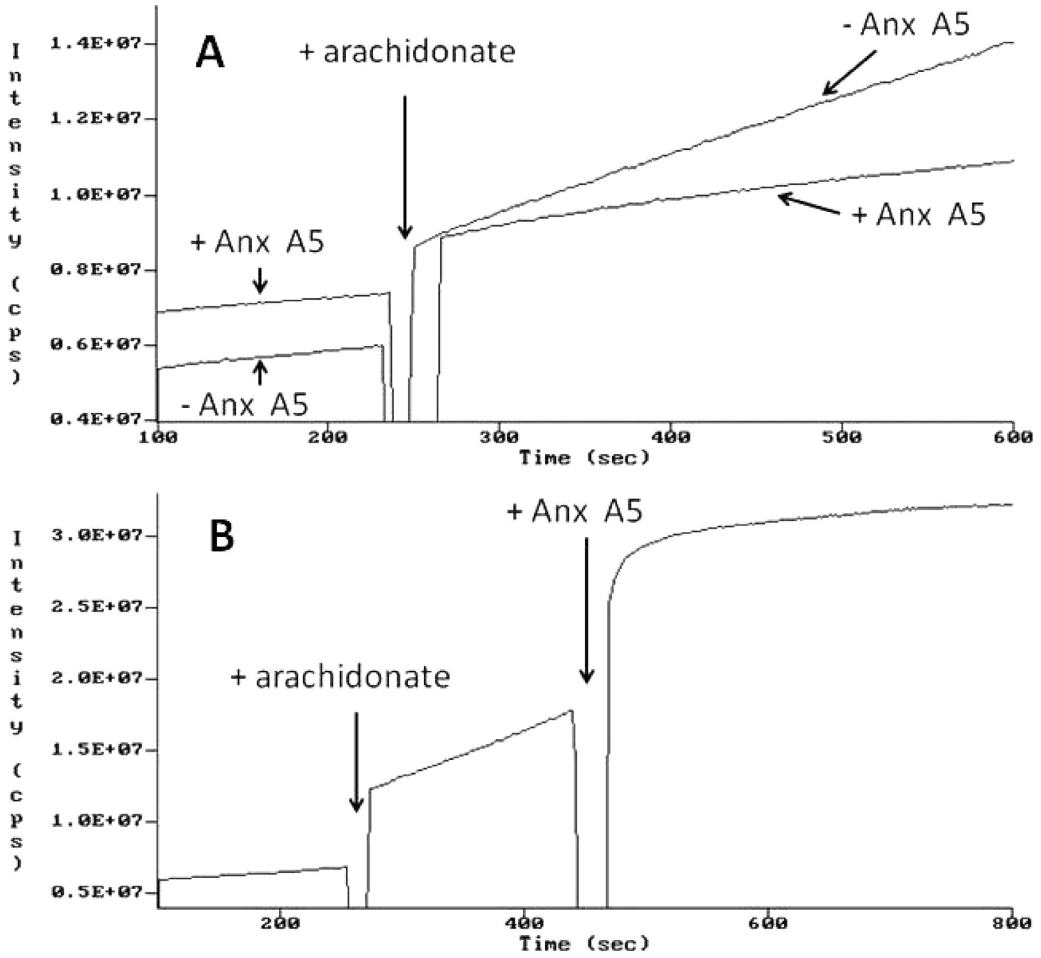

Figure 2. Annexin A5 protects membranes from leakage due to arachidonic acid. (A) Liposomes were preincubated in assay buffer with 5 mM $MgCl_2$ (−Anx A5). At 250 s, 2 μL of 0.4 mg/mL arachidonic acid dissolved in ethanol was added, giving a final arachidonic acid concentration of 2.7 μg/mL (+arachidonate). This resulted in a burst of release of CF followed by continued leakage of CF at an increased rate as shown. When the liposomes were preincubated with 4.8 μg/mL A5 (+Anx A5), the initial burst of CF release and the subsequent increase in the release rate were inhibited as shown. (B) Liposomes were preincubated in the absence of annexin A5. At 250 s, 2.7 μg/mL arachidonic acid was added, causing an increase in the rate of CF release. At 450 s, 4.8 μg/mL annexin A5 was added, which resulted in a burst of CF release followed by a suppression of the release rate to a level comparable to the initial rate.

leak during the course of the experiments. Calcium was tested at a single concentration of 1 mM, reflecting the high levels of calcium that might be anticipated at a site of damage at the plasma membrane of a cell (or the membrane of a calcium-containing organelle). In some cases, high concentrations of the added permeabilizing agents promoted vesicle aggregation; therefore, the leakage measurements were limited to lower concentrations of the agents at which this was not detected in turbidity measurements. Some poorly soluble agents were added in ethanol or DMSO as a solvent. The amounts of these solvents were kept to a minimum to reduce the level of leakage due to the solvent. Leakage rates due to the solvent are reported in the experiments below if they were above baseline leakage in the absence of solvent.

Effects of Annexins on Baseline Leakage of CF from Liposomes. When incubated at 37 °C in 100 mM KCl, 50 mM HEPES-NaOH (pH 7.4), and 1 mM $CaCl_2$ (assay buffer), the liposomes exhibited a baseline leakage of CF that was enhanced by increasing the concentration of Mg^{2+} (Figure 1). When the liposomes were added to the cuvette with the annexin already present in solution in the cuvette, the slopes of the CF release curves were analyzed after 1−2 min (Figure 1A). At 1 mM Mg^{2+}, the rate of leakage was low and only slightly affected by the binding of either annexin, which caused a 0−10% decrease in slope during the time course of the experiment (up to 20 min). At 3.5 mM Mg, binding of annexin A5 resulted in a very significant 43% reduction in the rate of leakage (Figure 1A) and annexin A6 a more modest 12% reduction in the rate of leakage. At 5 mM Mg^{2+}, annexin A5 caused a 42% reduction in the slope; the effect of annexin A6 was not determined at this Mg^{2+} concentration.

When the liposomes were preincubated in the cuvette first and then the annexin was added, it was possible to observe a burst of release of CF apparently caused by the initial binding of the annexin to the liposome membrane (Figure 1B). Subsequent to this event, the rate of release was reduced by the presence of the annexin. In the experiment illustrated in Figure 1B, annexin A5 reduced the rate of release by 11% at 1 mM Mg, 65% at 3.5 mM Mg, and 84% at 5 mM Mg. The size of the initial burst of CF release caused by the binding of the annexin, as seen in the figure, was larger in the presence of higher Mg^{2+} concentrations: This burst of CF release in 3.5 mM Mg^{2+} was 8 times greater than at 1 mM Mg^{2+}, and 12 times higher at 5 mM Mg^{2+} than at 1 mM Mg^{2+}.

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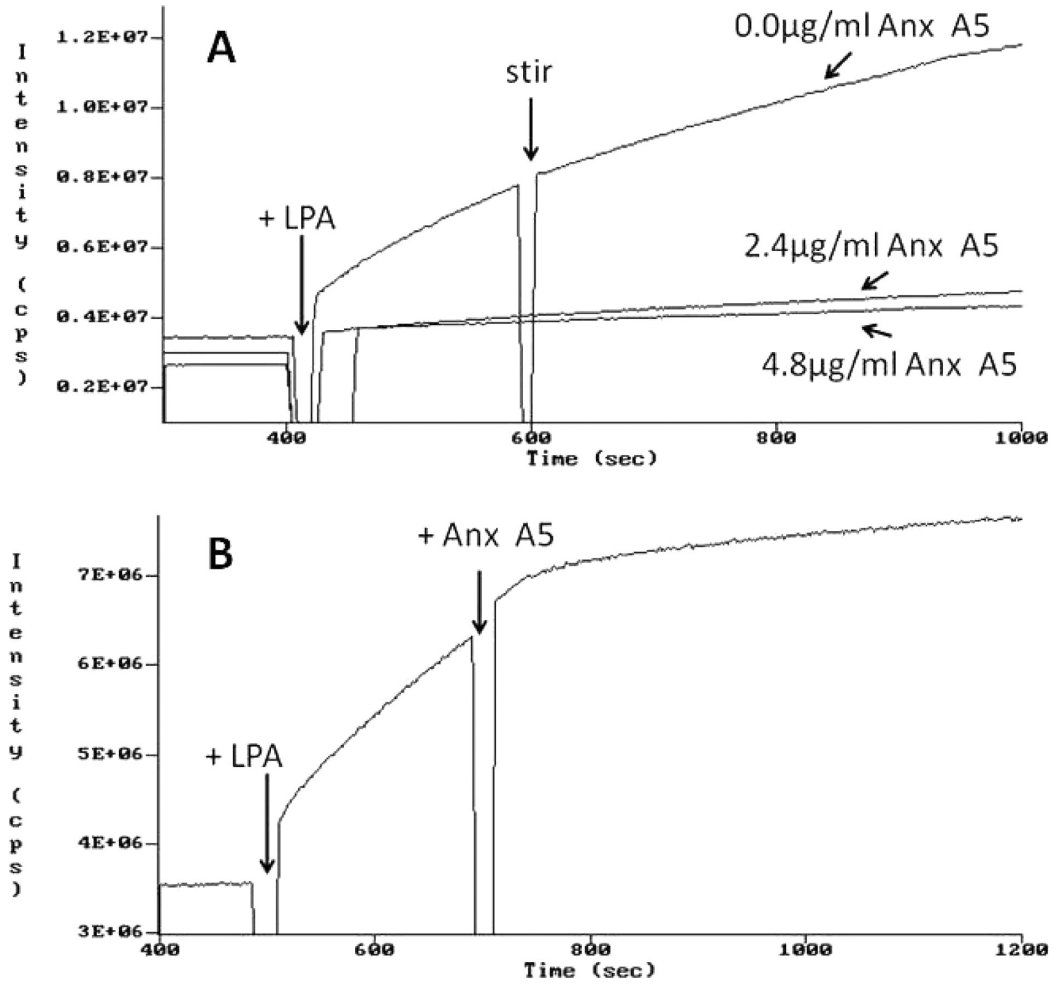

Figure 3. Annexin A5 inhibits the increase in the rate of CF leakage from liposomes due to addition of 1-palmitoyllysophosphatidic acid (LPA). (A) LPA in 10 μL of water was added to liposomes in assay buffer with 1 mM $MgCl_2$ at 450 s (+LPA, final LPA concentration of 6.7 μg/mL), causing an increase in the rate of CF release. When the liposomes were preincubated with 2.4 or 4.8 μg/mL annexin A5, as marked, both the initial burst of CF release and the subsequent increase in the rate of release due to LPA were inhibited. At 600 s, the LPA alone sample was stirred (stir) as a control for the effects of stirring (compare with the annexin addition at 700 s in panel B). (B) After addition of 6.7 μg/mL LPA at 500 s (+LPA), 4.8 μg/mL annexin A5 was added at 700 s (+Anx A5), which reduced the rate of CF leakage.

When liposomes were added to assay buffer containing very high concentrations of divalent cations (10 mM Ca^{2+} and 10 mM Mg^{2+} together), the liposomes rapidly aggregated giving a large increase in turbidity (absorbance at 350 nm) of 300% over 6 min and the CF was completely released during the first minute. At 1 mM Mg and 1 mM Ca, no turbidity increase was seen. However, at both 3.5 and 5 mM Mg^{2+} in the presence of 1 mM Ca, a small increase in turbidity was detectable (initial rate of 0.026% s^{-1}, compared to 10% s^{-1} in the presence of 10 mM Ca and 10 mM Mg), suggesting a slow process of vesicle aggregation may have been occurring, although other changes in membrane organization such as divalent cation-induced lipid phase separation may also have contributed to this small increase in turbidity. To determine whether the increased rate of baseline CF efflux at the higher Mg^{2+} concentrations was dependent upon vesicle−vesicle aggregation, the release rate was analyzed as a function of the vesicle concentration, anticipating that the rate would be second-order relative to vesicle concentration if vesicle−vesicle interaction were required to promote the release of CF. However, with 5 mM Mg^{2+} and 1 mM Ca^{2+} in the buffer, doubling the vesicle concentration resulted in an increase in the rate of release by a factor of 1.91 ± 0.15 (average of two independent experiments), suggesting a first-order dependence on vesicle concentration. The higher Mg^{2+} concentrations (3.5 and 5 mM) therefore appeared to be directly influencing the intrinsic permeability of the liposome membranes.

Effects of Annexins on Disruption of the Membrane Permeability Barrier by Arachidonic Acid. Arachidonic acid is an important precursor for lipidic signaling molecules and is liberated by phospholipases from membrane phospholipids. As seen in Figure 2, when free arachidonic acid was added in ethanol as a vehicle it caused an increase in the liposome leakage rate (3.5-fold increase in the slope). An initial burst of release of CF occurred when the arachidonate was added, but a burst of similar magnitude also occurred with an ethanol control and so may be due to a transient action of ethanol on a small number of vesicles before it is diluted. After this initial burst, there was no increase in the rate of leakage due to the ethanol alone above the initial baseline rate. When the ethanol/arachidonate stock mixture was diluted 3-fold with assay buffer immediately before the addition of the arachidonate, there was no increase in slope due to the arachidonate and the initial burst was also almost eliminated.

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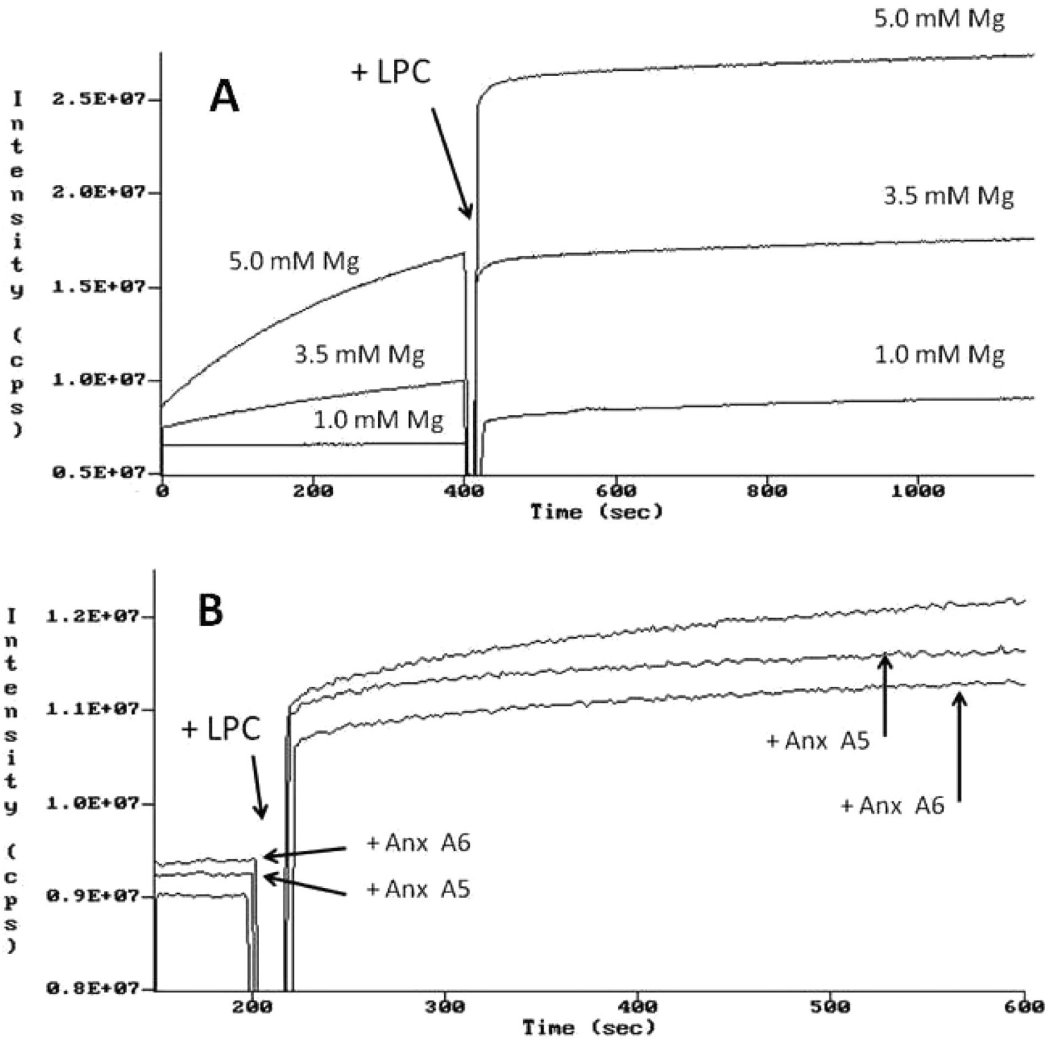

Figure 4. Effects of 1-oleoyl lysophosphatidylcholine (LPC) and annexins on liposome permeability. (A) LPC reduces liposome permeability at high magnesium concentrations. Liposomes were incubated in assay buffer with 1.0, 3.5, or 5.0 mM $MgCl_2$ as marked. At 400 s, LPC in 5 μL of water was added to give a final concentration of 16.7 μg/mL (+LPC). As shown, the rate of release of CF was increased by LPC at 1 mM $MgCl_2$ but reduced at the higher levels of $MgCl_2$. (B) Annexins A5 and A6 reduce the rate of leakage of CF from liposomes caused by the addition of LPC. Liposomes were incubated in assay buffer with 1 mM $MgCl_2$ in the absence of annexin or in the presence of 4.5 μg/mL annexin A5 (+Anx A5) or 4.3 μg/mL annexin A6 (+Anx A6). At 200 s, LPC was added to a final concentration of 8.4 μg/mL (+LPC).

Therefore, it appears that if the arachidonate is allowed to form micelles in buffer before addition to the liposomes its transfer to the liposome membrane may be blocked on the time scale of the experiment.

When the liposomes were preincubated with 4.8 μg/mL annexin A5, the rate of leakage due to the addition of arachidonate was strongly suppressed as shown in Figure 2A. Similarly, when the same amount of annexin A5 was added after the arachidonate, it caused an initial burst of release of CF and then strongly inhibited the leak, reducing it to a rate similar to that seen before addition of the fatty acid (Figure 2B).

Annexin A6, however, added before the arachidonate at a similar concentration (4.9 μg/mL) caused a 24% enhancement of the rate of leakage due to arachidonic acid. At a 2-fold higher concentration of annexin A6 (9.8 μg/mL), this stimulating effect on leakage was lost, but no protection from the permeability loss due to arachidonate was observed with annexin A6 at any protein concentration. If added after the arachidonate, 4.9 μg/mL annexin A6 also caused a 38% enhancement in the CF release rate.

Effects of Annexins on Disruption of the Membrane Permeability Barrier by Lysophosphatidic Acid (LPA). Lysolipids such as lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA) are generated by the action of phospholipase A2 in the process of liberating arachidonic acid as a precursor to prostaglandin and leukotriene signaling molecules. Both agents have significant detergent-like properties. As illustrated in Figure 3A, the addition of 1-palmitoyl LPA to liposomes promoted an initial burst of release of CF followed by a high rate of continued release. Similar to its action with arachidonic acid, annexin A5 was found to virtually completely protect the liposomes from this leakage, whether added before or after LPA (Figure 3A,B).

In contrast to the results with annexin A5, when annexin A6 under these conditions was bound to the liposomes it caused a 3.6-fold greater initial burst of CF release when the LPA was added, and a 2.8-fold elevated leakage rate subsequent to the initial burst. When annexin A6 was added after the LPA, annexin A6 also caused a slight increase (1.2-fold) in the release rate.

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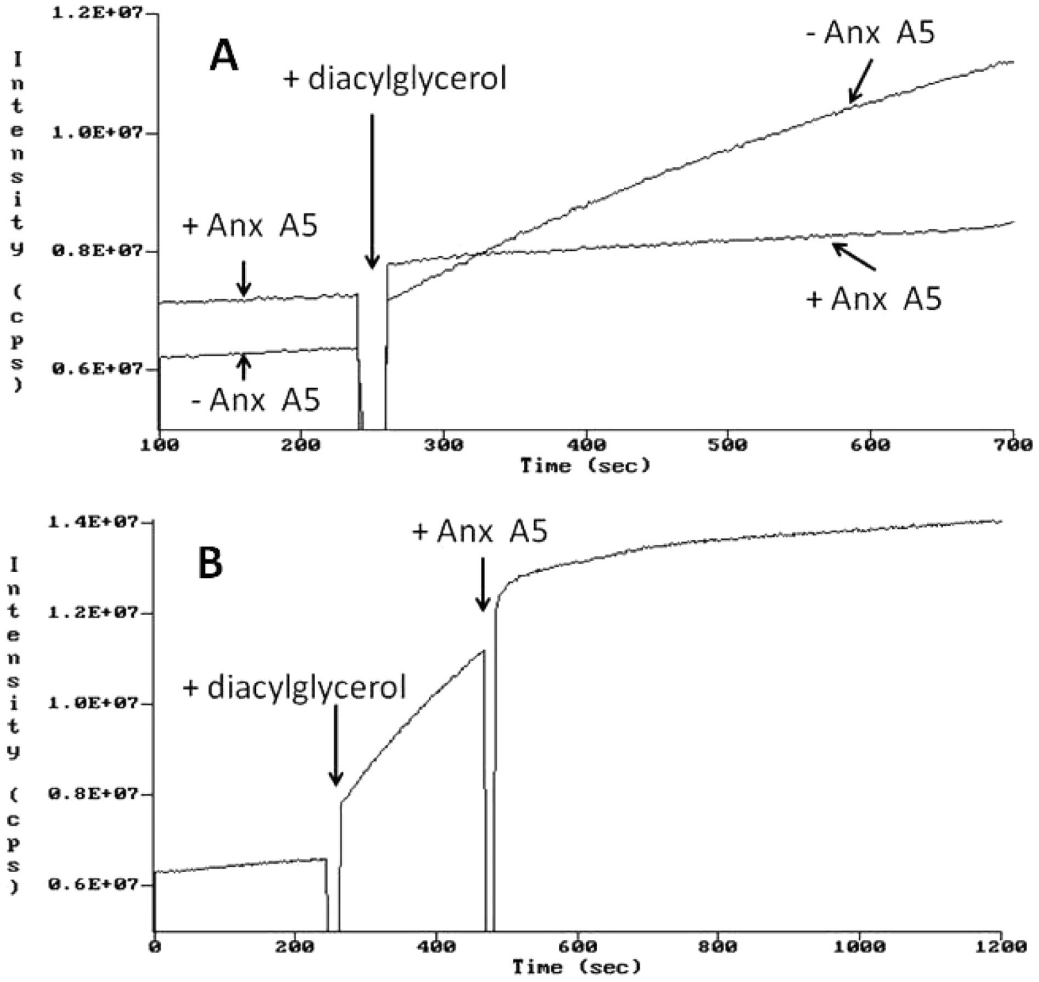

Figure 5. Annexin A5 inhibits membrane leakage induced by diacylglycerol (diolein). Liposomes were incubated in assay buffer with 3.5 mM $MgCl_2$. (A) At 250 s, diacylglycerol dissolved in 3 μL of ethanol was added to give a final concentration of 1.0 μg/mL (+diacylglycerol). In one sample, the vesicles were preincubated with 4.8 μg/mL annexin A5 (+Anx A5), which blocked the increase in the rate of leakage after the initial burst of CF release that occurred when the diacylglycerol and ethanol were added. (B) At 250 s, diacylglycerol was added as in panel A to liposomes incubated in the absence of annexin. At 450 s, annexin A5 was added to a final concentration of 4.5 μg/mL (+Anx A5). After a burst of release of CF associated with the binding of the annexin to the liposomes, the subsequent rate of CF leakage was reduced to the baseline release rate in the absence of diacylglycerol.

Effects of Annexins on Disruption of the Membrane Permeability Barrier by Lysophosphatidylcholine (LPC). In contrast to LPA, LPC is zwitterionic and has a net neutral charge. It has been studied extensively for its effects on model membrane structure and has been assigned roles in membrane permeabilization and membrane fusion.[30−33] In these experiments, the addition of 1-oleoyl LPC in aqueous buffer or ethanol had only modest effects on liposome permeability at 1 mM Mg^{2+}, acting to slightly enhance leakage (Figure 4A). At higher Mg^{2+} concentrations, it appeared that larger amounts of LPC were able to interact with and/or enter the lipid bilayer. The initial interaction was associated with a large and rapid release of CF followed by stabilization of the membrane as CF permeability was then reduced below the original baseline (Figure 4A). This may have been due to a reduction of the surface charge of the bilayer as the neutral lipid was incorporated and to an alteration of lipid domain structure.

To establish a model for determining whether the annexins could protect against membrane permeabilization by LPC, attention was therefore focused on experiments with the lower concentration of Mg^{2+} (1 mM) in which the addition of LPC enhanced membrane leakage rather than reducing it. When preassociated with the liposomes, both annexins A5 and A6 reduced the initial burst of CF release associated with mixing LPC with the liposomes and caused a 44% (A5) or 38% (A6) reduction in the leakage rate (Figure 4B). If the annexins were added after LPC, the leakage rate was reduced by 28% by annexin A5 and 51% by annexin A6.

Effects of Annexins on Disruption of the Membrane Permeability Barrier by Diacylglycerol. Diacylglycerols are important intermediates in the biosynthesis and degradation of triglycerides, glycerophospholipids, and glyceroglycolipids and are known to act as second messengers in cell signaling through the activation of protein kinase C. Diacylglycerol has been found to cause alterations in membrane curvature, modification of surface charge, and promotion of bilayer to nonbilayer phase transitions.[31,34] The addition of diacylglycerol (diolein) to the liposomes from an ethanol stock solution in the presence of 3.5 mM Mg^{2+} caused a significant, sustained leakage of CF (Figure 5). When annexin A5 was prebound to the liposomes, it effectively blocked the increase in the rate of release of CF caused by the diacylglycerol after an initial burst of release that

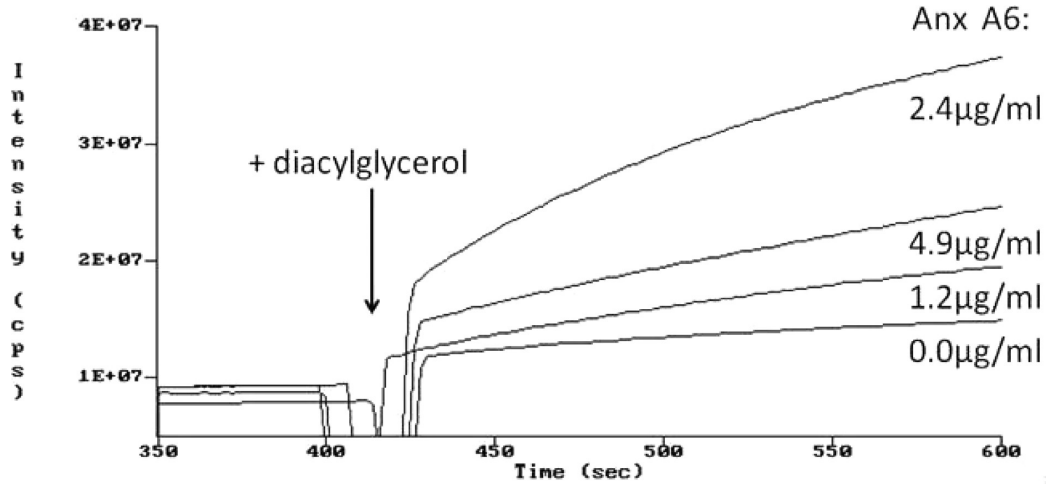

Figure 6. Annexin A6 prebound to the liposomes enhances the leakage caused by the addition of diacylglycerol (diolein) in a biphasic manner. Liposomes were preincubated with annexin A6 at the concentrations indicated. At 400 s, 3 μL of a diacylglycerol stock solution in ethanol was added to give a final diacylglycerol concentration of 1.0 μg/mL (+diacylglycerol).

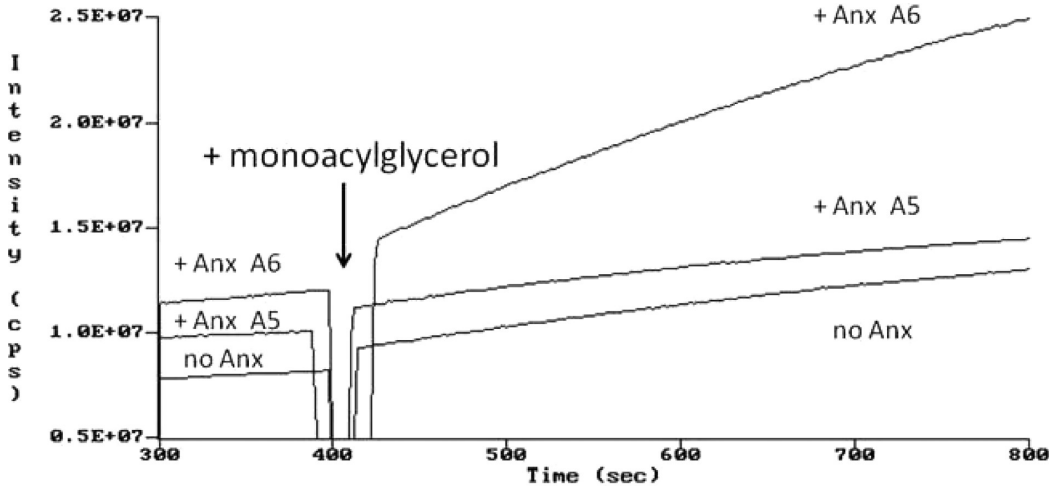

Figure 7. Effects of annexins A5 and A6 on membrane permeabilization by monoacylglycerol. Liposomes were incubated in assay buffer with 3.5 mM $MgCl_2$. At 400 s, monoacylglycerol (monoolein) was added in 5 μL of ethanol to a final concentration of 5.0 μg/mL (+monoacylglycerol). Preincubation of the liposomes with 4.5 μg/mL annexin A5 (+Anx A5) reduced the rate of leakage and with 4.3 μg/mL annexin A6 (+Anx A6) increased the rate of leakage compared to that with monoacylglycerol alone (no Anx).

may have been due in part to the ethanol vehicle (Figure 5A). When the diacylglycerol was added first, annexin A5 was effective in blocking the diacylglycerol-dependent leak, although the initial interaction of the annexin with the membrane caused a small burst of CF release before the release rate was returned to baseline levels (Figure 5B).

In contrast, annexin A6 at all levels that were tested caused an enhancement of the release of CF due to diacylglycerol, although the effect was biphasic in that increasing the annexin A6 concentration increased the release rate up to an annexin concentration of 2.5 μg/mL, but a lower release rate was promoted by 4.9 μg/mL annexin (Figure 6).

Effects of Annexins on Disruption of the Membrane Permeability Barrier by Monoacylglycerol. Monoacylglycerols are generated through the action of lipase on triglycerides[35] and are also present as specific endocannabinoids such as 2-arachidonoylglycerol that function as signaling molecules.[36] Similar to the effects of annexins on diacylglycerol-treated liposomes, annexin A5 slightly inhibited CF leakage caused by monoacylglycerol (monoolein) and annexin A6 exacerbated the leakage caused by monoacylglycerol (Figure 7).

Effects of Annexins on Disruption of the Membrane Permeability Barrier by Spermidine. The naturally occurring polyamines spermine, spermidine, and putrescine are polycations that are found at levels as high as millimolar in many cell types. Because of their cationic character, polyamines bind to nucleic acids and also interact with anionic phospholipids in cell membranes. Some effects of polyamines on membrane properties have been described, including membrane stabilization against osmotic stress, changes in membrane fluidity, changes in electrical conductivity, and effects on divalent cation-induced fusion of liposomes.[37−40]

Annexin A5 prebound to the liposomes was highly effective at preventing leakage caused by 2 mM spermidine in the presence of 1 mM Mg, reducing the rate of leakage to baseline levels at 4.5 μg/mL annexin, and inhibiting leakage by 50% between 0.3 and 0.6 μg/mL (Figure 8A and Table 1). When annexin A5 was added after the spermidine, annexin A5 also suppressed leakage to baseline levels, but only after causing an

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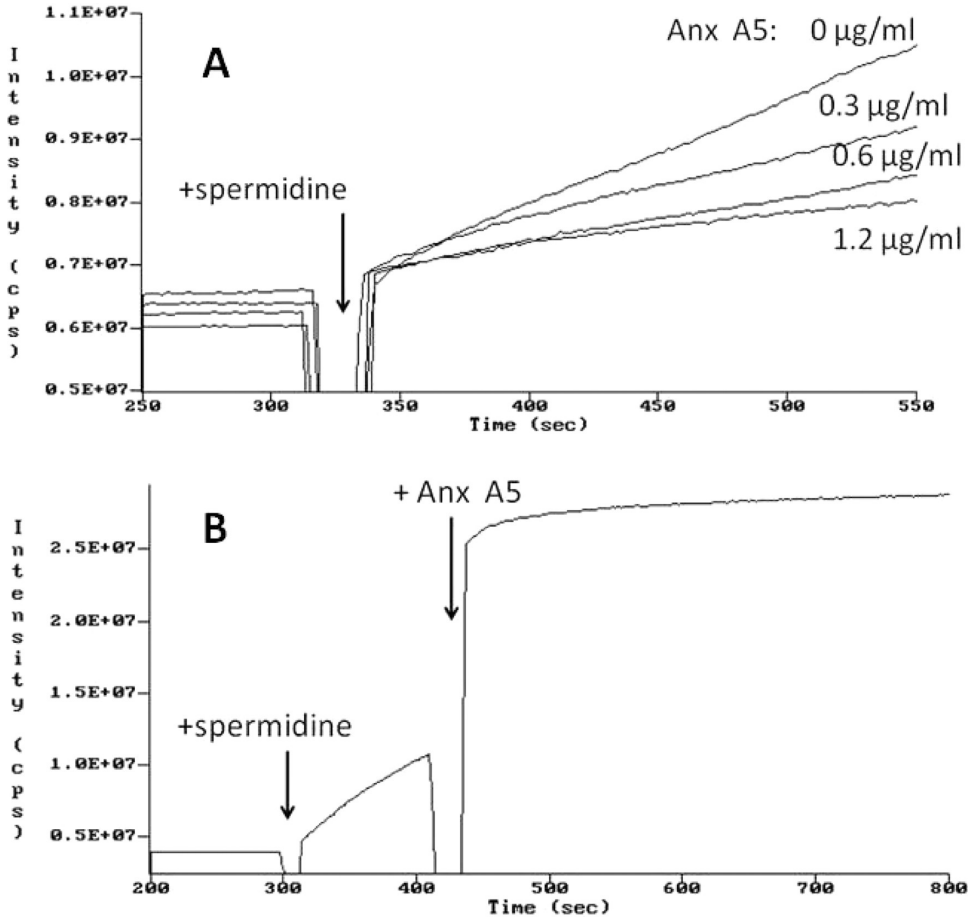

Figure 8. Annexin A5 suppresses CF leakage due to spermidine. (A) Liposomes were incubated in assay buffer with 1 mM $MgCl_2$ and with the indicated concentrations of annexin A5. At 325 s, spermidine in water was added to a final concentration of 2 mM (+spermidine). Slopes of the traces after the addition of spermidine are listed in Table 1. (B) Addition of annexin A5 after spermidine caused a burst of CF release and then stabilized the membrane leak. Spermidine was added at 300 s to a final concentration of 2 mM (+spermidine). Annexin A5 was added at 425 s to a final concentration of 1.9 μg/mL (+Anx A5).

Table 1. Effects of Annexins A5 and A6 on the Rates of Release of CF from Liposomes Treated with Spermidine[a]

[annexin A5] (μg/mL)	relative slope	annexin A6 (μg/mL)	relative slope
0.0	100	0.0	100
0.3	57.4	4.3	40.8
0.6	41.5		
1.2	28.4		
4.8	1.9		
0.0 (no spermidine)	1.5		

[a]The spermidine concentration was 2 mM; other conditions are described in Materials and Methods and the legend of Figure 8. The slopes of fluorescence vs time curves were normalized to the value in the presence of spermidine but in the absence of annexins.

abrupt and significant burst of CF release during the binding of the annexin to the liposomes (Figure 8B). This initial burst of CF release due to the initial binding of the annexin was much greater than that seen with liposomes in the absence of spermidine at 1 mM Mg^{2+} (compare with Figure 1B).

In similar experiments, annexin A6 also reduced the rate of leakage due to spermidine in the presence of 1 mM Mg^{2+}, although it was less effective. If added before 2 mM spermidine at a concentration of 4.3 μg/mL, annexin A6 reduced the rate of leakage due to spermidine by 59.2%, and if added after the spermidine at the same concentration, it reduced the rate of leakage by 57.1%. The binding of annexin A6 to the liposomes did not cause the significant burst of release of CF as seen with annexin A5 in Figure 8B.

The addition of 2 mM spermidine under these conditions (1 mM Mg^{2+}) was found to cause a slow increase in the turbidity of the liposome suspension at a rate of 0.05% s^{-1} relative to the initial turbidity of the vesicle suspension. This rate of turbidity increase was 200-fold less than the rate of turbidity increase seen when the vesicles were incubated in a mixture of 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (see the section above on baseline release rates). This slow turbidity increase may have been due to vesicle aggregation caused by interaction of the positively charged spermidine with the negatively charged vesicles, or possibly reorganization of lipid domains in the membrane. To determine if vesicle aggregation may have contributed to the release of CF, two independent experiments with different vesicle preparations were performed to determine the dependence of the rate of CF leakage upon vesicle concentration, similar to the strategy used to check for effects of vesicle concentration on baseline leakage. Doubling the amount of vesicles in the assays resulted in a 2.08 ± 0.61-fold increase in the rate of release of CF, indicating a first-order dependence of the release rate on vesicle concentration. This suggests the spermidine was acting directly to alter the intrinsic permeability

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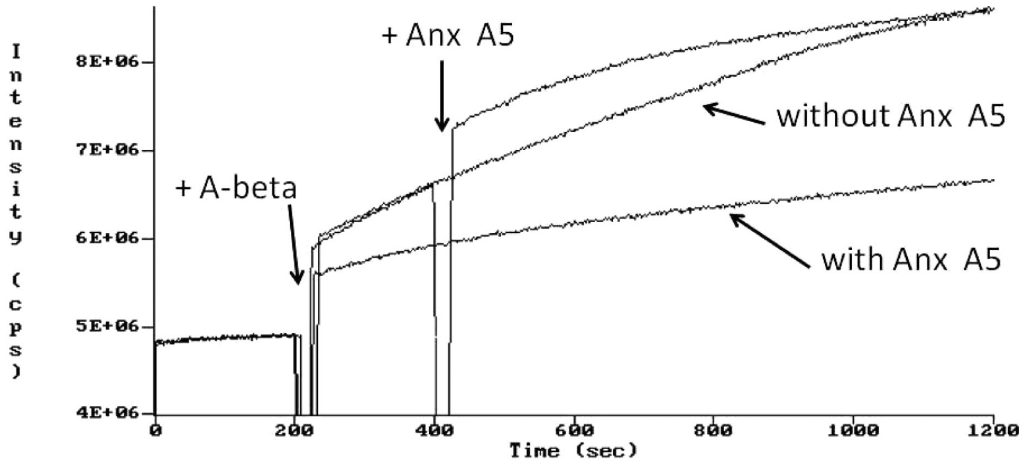

Figure 9. Annexin A5 protects liposomes from permeabilization by A-β. Liposomes were incubated in assay buffer with 1 mM $MgCl_2$. At 200 s, A-β was added in 7 μL of DMSO (+A-beta) to a final peptide concentration of 12.8 μM and stimulated CF efflux (without Anx A5). When 4.8 μg/mL annexin A5 was incubated with the liposomes prior to the addition of A-β, the rate of CF release due to A-β was reduced (with Anx A5). When the same amount of annexin was added after A-β (+Anx A5 at 400 s), the rate of CF release was subsequently reduced to the rate seen when the protein was added before the A-β peptide.

of the membranes in a manner that was independent of vesicle aggregation.

Effects of Annexins on Disruption of the Membrane Permeability Barrier by Amyloid-β Peptide 1−42 (A-β). Alzheimer's disease is characterized by the buildup of fragments of the amyloid precursor protein, a neuronal plasma membrane protein, in extracellular spaces in the brain.[41] Soluble oligomers of the fragment termed amyloid-β (A-β) have been demonstrated to be toxic to neurons, so it has been hypothesized that A-β contributes to the pathology of the disease.[42] A body of evidence suggests A-β exerts its toxic effects through damaging the neuronal cell membrane and allowing excess calcium to enter nerve cells.[43−45]

As shown in Figure 9, A-β (12.8 μM) increased the rate of release of CF from the liposomes and annexin A5 reduced this A-β-dependent leakage whether it was added to the liposomes before or after A-β. The A-β peptide was added to the vesicles in DMSO as a solvent. Control experiments indicated that DMSO alone had small but significant effects on the leakage rate and that this was suppressed by the annexin as well (Table 2). The effectiveness of the annexin had a very sharp dose−response titration providing no protection at 2.4 μg/mL and maximal protection at 3.6 μg/mL.

Annexin A6 also inhibited the release of CF due to A-β (5.5 μM), but with a very dramatic anomaly at an annexin concentration of 4.9 μg/mL at which the annexin enhanced release due to A-β (Figure 10). Annexin A6 inhibited the rate of CF release due to A-β by up to 50% before this anomaly; at the anomaly, it caused a large burst of CF release followed by a rate of leakage 13-fold greater than the rate of leakage due to A-β alone (Table 2). At concentrations above this anomalous point, annexin A6 again reduced the rate of leakage due to A-β by 50%. This anomalous effect of annexin A6 was seen if the annexin was added before or after A-β (Figure 10B). Annexin A6 at this concentration (4.9 μg/mL) did not enhance baseline release in the presence of the vehicle DMSO alone.

Effects of Annexins on Disruption of the Membrane Permeability Barrier by Amylin. In type 2 diabetes, there is a buildup of insoluble, fibrillar deposits of the peptide hormone amylin in the vicinity of the β cells of the pancreas.[46] Soluble oligomers of amylin are apparently the precursors of these amylin fibrils and are themselves toxic to the β cells.[47−49] It has been hypothesized that these amylin oligomers contribute to the cause or severity of β cell destruction in diabetes by a mechanism similar to that proposed for the action of A-β on nerve cells, damaging the β cell plasma membrane and allowing excess calcium to enter the cells.

When amylin was added to the liposomes, it promoted the release of CF, and this release could be inhibited by annexin A5 whether the annexin was added to the liposomes before or after the addition of the peptide (Figure 11). At 4.8 μg/mL, annexin A5 inhibited the enhancement of release by 6.4 μM amylin by 84%. When the same concentration of annexin A5 was added after the amylin (Figure 11B), the enhancement of release due to the amylin was reduced by 69%. Annexin A6 also provided protection of the membrane from permeabilization by amylin,

Table 2. Effects of Annexins A5 and A6 on the Rates of Release of CF from Liposomes Treated with Amyloid-β[a]

annexin A5		annexin A6	
addition	relative slope	addition	relative slope
none	100	none	100
DMSO	125	DMSO	157
A-β	338	A-β	504
A-β with 1.2 μg/mL annexin A5	345	A-β with 1.2 μg/mL annexin A6	357
A-β with 2.4 μg/mL annexin A5	375	A-β with 2.5 μg/mL annexin A6	264
A-β with 3.6 μg/mL annexin A5	108	A-β with 3.7 μg/mL annexin A6	407
A-β with 4.8 μg/mL annexin A5	62	A-β with 4.9 μg/mL annexin A6	6507
DMSO with 4.8 μg/mL annexin A5	82	A-β with 9.9 μg/mL annexin A6	232

[a]Amyloid-β (A-β) was added at a final concentration of 5.5 μM from 3 μL of a DMSO stock. DMSO controls were performed with 3 μL of DMSO alone. Slopes were normalized to 100 for the baseline slope in the absence of DMSO or A-β. The slopes were determined 600−800 s after the addition of A-β (corresponding to 800−1000 s in Figure 9). The experiments with annexin A5 and annexin A6 were performed with different vesicle preparations. Other conditions were as described in Materials and Methods and the legend of Figure 9.

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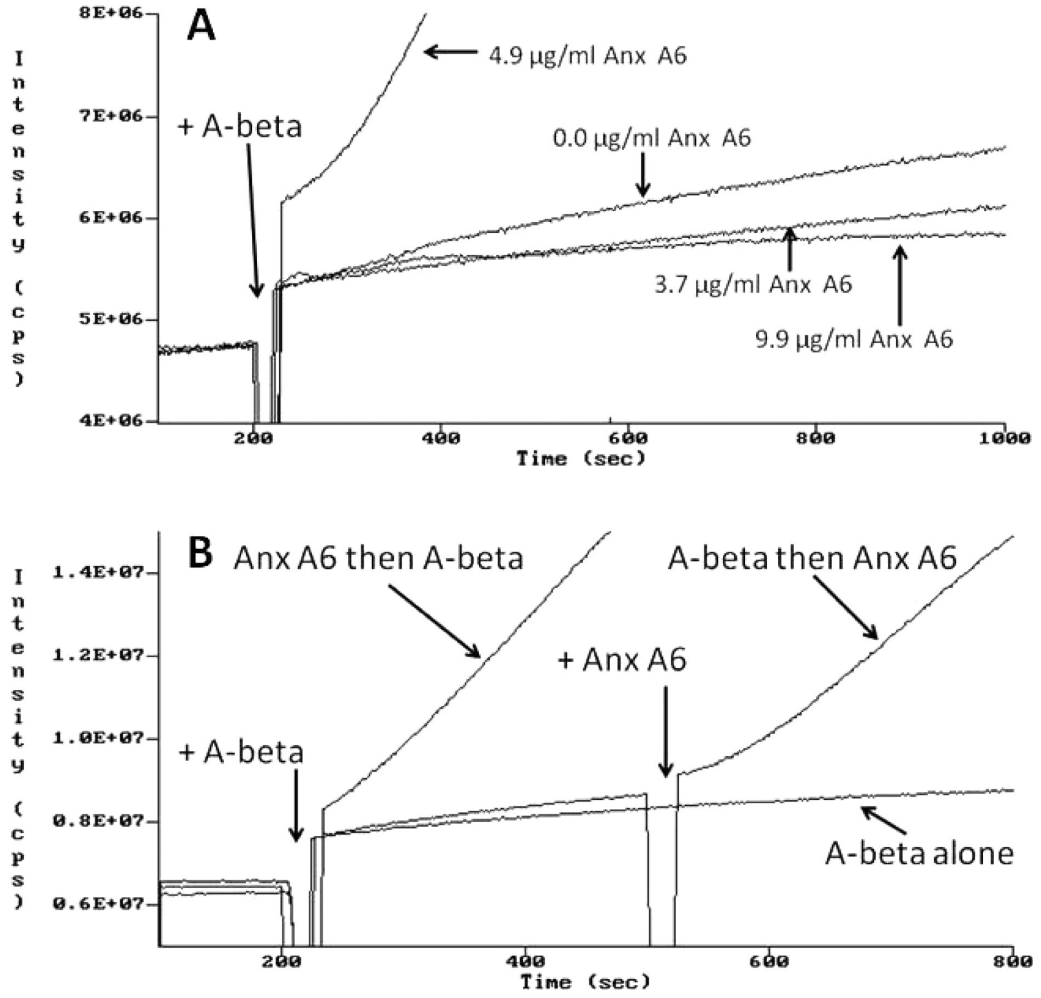

Figure 10. Effects of annexin A6 on CF leakage induced by A-β. Liposomes were incubated in assay buffer with 1 mM $MgCl_2$. (A) Liposomes were preincubated with 0, 3.7, 4.9, or 9.9 μg/mL annexin A6, and A-β was added at 200 s in 3 μL of DMSO (+A-beta) to a final peptide concentration of 5.5 μM. The top trace shows the enhanced leakage when the vesicles were incubated in 4.9 μg/mL annexin A6. The relative slopes of the traces are listed in Table 2. (B) Prior to 200 s, liposomes were preincubated in the absence (two samples) or presence (one sample) of 4.9 μg/mL annexin A6. At 200 s, A-β was added in 3 μL of DMSO to give a final peptide concentration of 5.5 μM (+A-beta) in all three samples. The sample containing annexin A6 exhibited a very high rate of release of CF (Anx A6 then A-beta). At 520 s, 4.9 μg/mL annexin A6 was added to one of the two samples without annexin to which A-β had previously added at 200 s (A-beta then Anx A6). Note that the annexin strongly enhanced the leakage of CF induced by A-β whether it was added before or after A-β.

but it was somewhat less effective than annexin A5: at 4.9 μg/mL annexin A6, the increase in the release rate due to 6.4 μM amylin was reduced by 32%. Annexin A6 did not promote an anomalous enhancement of CF release like that seen with A-β even though annexin A6 was used at a similar concentration under similar conditions, including the use of DMSO as a vehicle.

Effects of Annexins on Disruption of the Membrane Permeability Barrier by Osmotic Shock. The annexins were tested for the ability to suppress leakage due to osmotic shock of liposomes, a type of stress that may serve as a model for membrane disruption due to mechanical means like those that might occur in muscular dystrophies, spinal cord injury, or traumatic brain injury.

Liposomes were incubated in a small volume (15 μL) of buffer with 150 mM KCl for osmotic support (see Materials and Methods for details). After 200 s, 290 μL of isotonic buffer (assay buffer containing 3.5 or 5 mM Mg) or hypotonic buffer (the same buffer without KCl) was used to dilute the sample approximately 20-fold in the fluorometer cuvette, and the fluorescence was subsequently monitored continuously (Figure 12). When samples were diluted with isotonic buffer, there was no significant leakage of CF associated with the dilution and mixing. When samples were diluted with hypotonic buffer, there was a burst of release of approximately 17% of the encapsulated CF. Subsequent to this burst, there was an increase in the leakage rate relative to the control not exposed to the osmotic shock, suggesting that although the membrane resealed after the shock, the permeability remained altered to some degree (Figure 12A).

When annexin A5 was present in the initial incubation period (at an amount such that the final concentration after dilution was 4.5 μg/mL), the amount of release of CF during hypotonic shock was reduced to 9% of the encapsulated CF, a reduction of 47% from the amount of leakage during osmotic shock in the absence of the annexin (Figure 12A). Subsequent to the shock, the continued presence of the annexin reduced the rate of leakage of CF to the control level seen with unshocked liposomes (Figure 12A).

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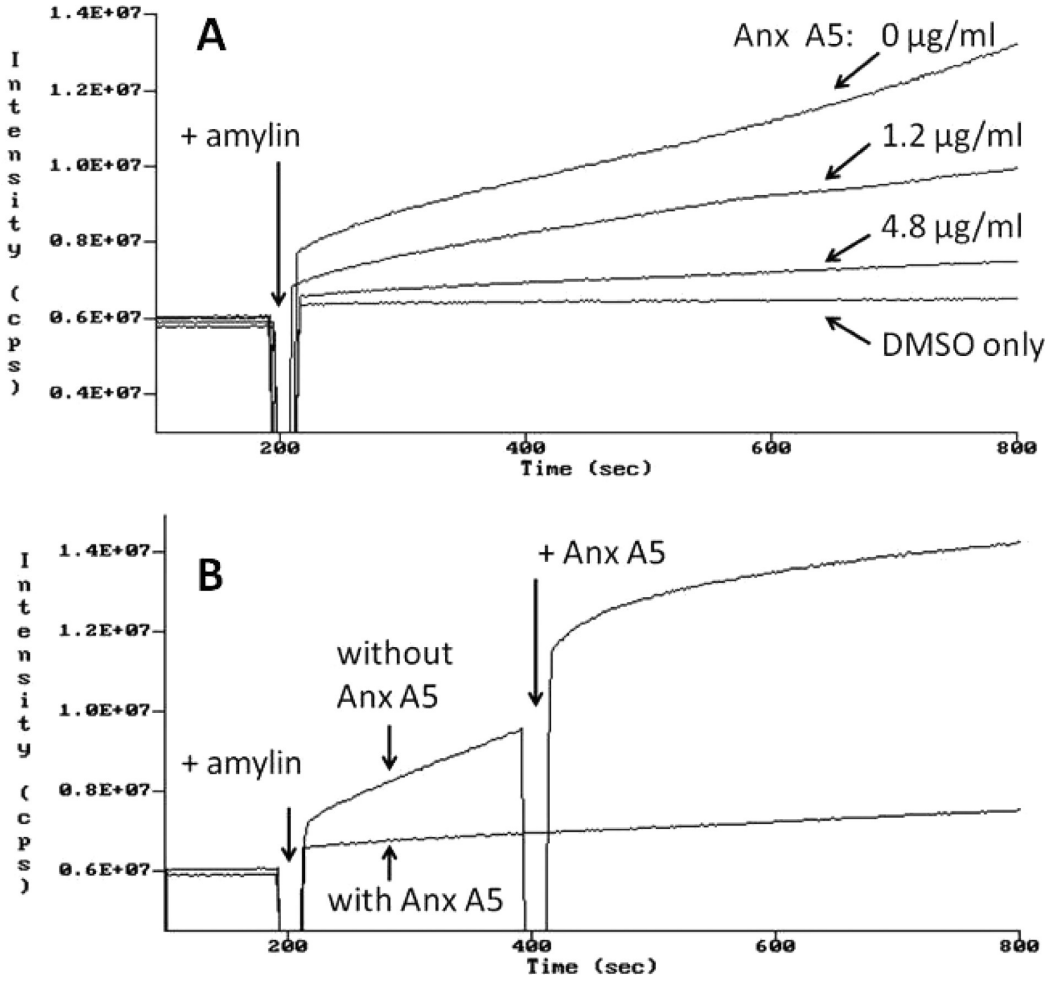

Figure 11. Annexin A5 inhibits leakage of CF induced by amylin. Liposomes were incubated in assay buffer with 1 mM $MgCl_2$. (A) Liposomes were preincubated with 0, 1.2, or 4.8 μg/mL annexin A5, as marked, and amylin was added at 200 s in 3 μL of DMSO to a final peptide concentration of 6.4 μM (+amylin). The trace marked DMSO only shows data for the sample without annexin but with 3 μL of DMSO alone added at 200 s. (B) Liposomes were preincubated in the absence (without annexin) or presence (with annexin) of 4.8 μg/mL annexin A5. At 200 s, amylin was added in 3 μL of DMSO to give a final concentration of 6.4 μM peptide (+amylin). At 400 s, 4.8 μg/mL annexin A5 was added to the sample initially without annexin.

Experiments performed with annexin A6 (4.3 μg/mL after dilution) also showed protection from osmotic shock, although the effect was weaker than with annexin A5 (Table 3). The degree of protection with annexin A6 was greater when the experiment was performed with 5 mM Mg^{2+} than with 3.5 mM Mg^{2+}, while the degree of protection with annexin A5 was similar at the two Mg^{2+} concentrations (Table 3). Because the osmotic shock occurred 200 s into the time course of the experiment but mixing was not complete and monitoring of fluorescence did not occur until approximately 220 s, Table 3 also includes data obtained by extrapolating the lines in the graphs to 200 s.

The ability of annexin A5 to provide protection against osmotic shock was calcium-dependent. When the osmotic shock experiment was conducted in the presence of EGTA instead of 1 mM $CaCl_2$, the presence of annexin A5 had no effect on the release of CF due to mixing with hypotonic medium (Table 3).

The protective action of annexin A5 occurred early in the process of osmotic shock. When the annexin was bound to the liposomes by the action of calcium in the preincubation step but the dilution was made with hypotonic buffer containing EGTA, which should promote the release of the annexin, 51% suppression of the leakage due to osmotic shock was observed (Figure 12B), although after the shock the rate of baseline release was not reduced by the annexin, as expected because of the removal of calcium (Figure 12B). However, annexin A6 was not effective in this regard as it caused a slight increase in the rate of release of CF that was not statistically significant upon osmotic shock with an EGTA-containing buffer. The failure of annexin A6 to provide protection in this case may reflect an off rate for annexin A6 when the calcium was removed that is faster than that with annexin A5.

When the initial incubation was performed in 1 mM EGTA instead of 1 mM $CaCl_2$ and the dilution was performed in medium containing 1 mM $CaCl_2$, there was no protection by annexin A5 from the osmotic shock, although the subsequent slow rate of leakage was reduced by the annexin. Apparently, the annexin was unable to move to the membrane quickly enough when the calcium-containing medium was added to provide protection from the initial impact of the osmotic shock.

Negative Controls. Disruption of the liposome permeability barrier by the amphiphilic small molecules studied here is likely to have been due to their ability to disrupt the order of

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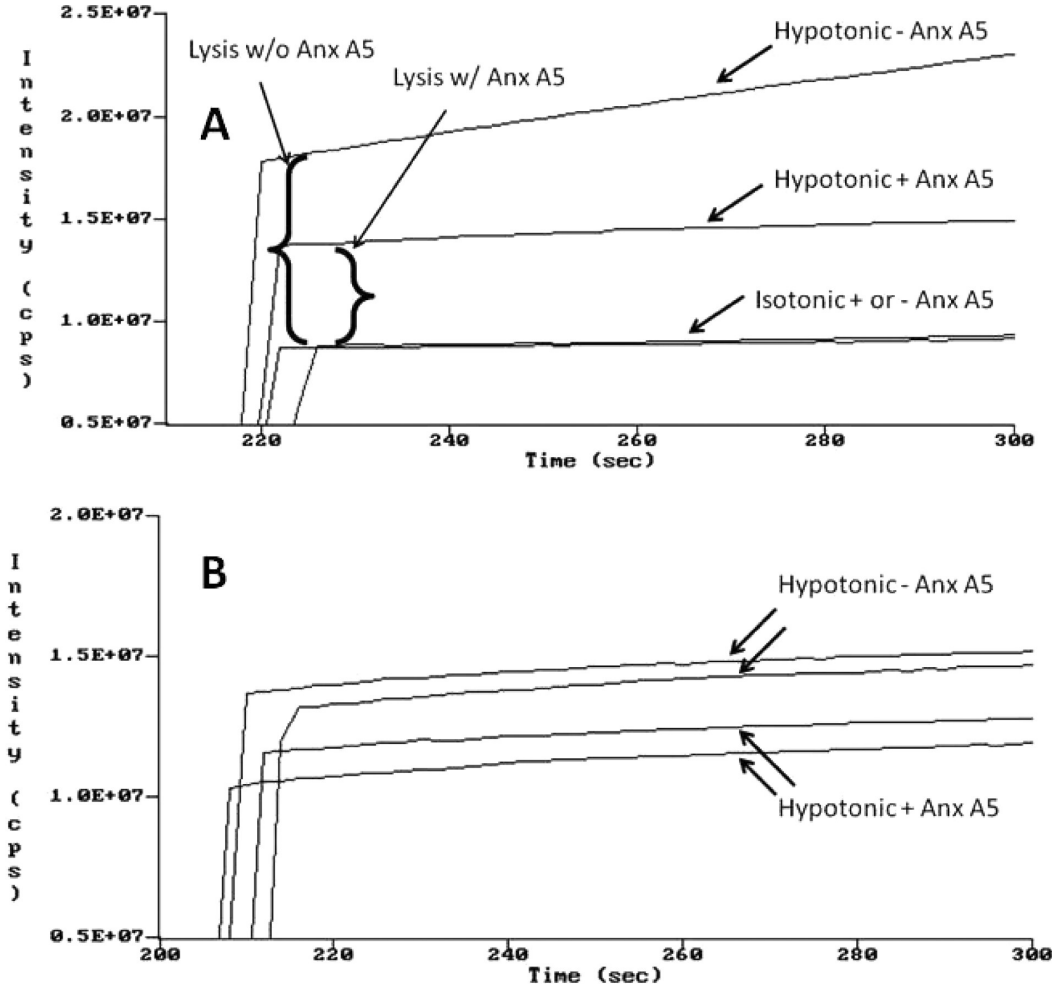

Figure 12. Annexin A5 suppresses the release of CF from liposomes exposed to osmotic shock. (A) Liposomes with or without annexin A5 were incubated in a small volume (15 μL) for 200 s and then were diluted to a volume of 305 μL with isotonic or hypotonic buffer (see the text for details). After the mixing had been completed at approximately 220 s, the fluorometer shutters were opened and the fluorescence was monitored continuously. Nearly coincident trace labeled Isotonic + or − Anx A5 represents liposomes incubated with or without annexin A5 (final concentration of 4.5 μg/mL after dilution) and mixed with isotonic buffer. For trace Hypotonic − Anx A5, lipsomes were incubated without annexin A5 with hypotonic buffer; for trace Hypotonic + Anx A5, lipsomes were incubated with annexin A5 with hypotonic buffer. Brackets mark the amount of CF released during the osmotic shock with or without annexin A5. The fluoresence intensity of suspensions after the addition of Triton was 6.7 × 10^7 cps. (B) Annexin A5 provides protection against osmotic shock at an early time point. Liposomes were incubated in a small volume with or without annexin A5 and then diluted with hypotonic buffer containing 3.5 mM $MgCl_2$ and 2 mM EGTA in place of calcium. For trace Hypotonic − Anx A5, duplicate samples lacked annexin A5; for trace Hypotonic + Anx A5, duplicate samples contained annexin A5. The fluorescence intensities at 220 s without annexin were (1.35 ± 0.03) × 10^7 cps and with annexin (1.10 ± 0.09) × 10^7 cps. The baseline intensity when diluted with isotonic buffer (not shown in the figure) was (0.86 ± 0.03) × 10^7 cps.

Table 3. Effects of Annexins A5 and A6 on the Amount of Release of CF from Liposomes Subjected to Osmotic Shock

	$[Mg^{2+}]$ (mM)	reduction in lysis[a] (%)	reduction extrapolated to 200 s[b] (%)
A5	3.5	46.9	42.1
A5	5.0	47.3	36.0
A5 without Ca^{2+}	3.5	0	0
A6	3.5	16.5	7.8
A6	5.0	28.6	23.4

[a]The percent reduction in lysis was determined by comparing the differences marked by the vertical brackets in the example in Figure 12. [b]The percent reduction extrapolated to 200 s was determined by extending the fluorescence traces, as seen in the example in Figure 12, to the time of initial mixing of the liposomes with hypotonic medium.

the lipid bilayer by acting as detergents. The action of spermidine on the other hand may have been due to the interaction of this positively charged compound with the negative charge on phosphatidylserine. As negative controls for these disruptive actions of the small molecules, several hydrophilic and/or negatively charged compounds were tested for their ability to promote leakage of CF from the liposomes. Glucose (10 mM), AMP (2 mM), ATP (2 mM), and FCCP (200 μM) were all inactive in promoting leakage. The inactivity of FCCP is of particular interest as it is a commonly used uncoupler, a proton ionophore,[50,51] that readily crosses the bilayer but as evidenced here does not cause sufficient disruption of bilayer structure to allow CF to permeate.

In addition, several unrelated proteins were tested as negative controls for the protective activity of the annexins: bovine serum albumin (defatted), chicken ovalbumin, soybean trypsin inhibitor, carbonic anhydrase, and lysozyme. The abilities of

these proteins to reduce the rate of baseline leakage and protect the liposome membranes subjected to three different types of challenge were determined: treatment with LPA, spermidine, and osmotic shock. The control proteins were tested at a concentration of 20 μg/mL; the maximal protective effects of the annexins were observed at concentrations of $\leq$5 μg/mL. Ovalbumin, trypsin inhibitor, and carbonic anhydrase provided no protection to the membranes. Lysozyme increased the baseline rate of release of CF from the liposomes and was not studied further. This action of lysozyme may be due to its high positive charge (calculated isoelectric point of 9.2) that may have promoted its binding to the membranes and disrupted the organization of the PS, similar to the action of spermidine. Serum albumin did not reduce the rate of baseline leakage or provide protection against osmotic shock. Serum albumin did, however, reduce the rate of leakage due to LPA and spermidine by 13 and 40%, respectively, when it was added to the liposome suspension before the lytic agents, but it is likely albumin bound these small molecules, therefore reducing their effective concentrations. When serum albumin was added after the lytic agents had already established an increase in the CF leakage rate, serum albumin did not inhibit leakage.

DISCUSSION

Membrane Disruption and Protection. Annexin A5, and to a lesser extent annexin A6, was found in this study to stabilize the permeability barrier of complex liposomes against a wide variety of stresses, as summarized in Table 4. The molecular nature of the membrane disruptions caused by the various stressing agents used has not been well characterized previously, particularly in a complex, four-component system such as that used here comprised of PC, PS, PE, and cholesterol. The mechanisms of membrane disruption by these agents may be diverse. High concentrations of Mg^{2+} or the positively charged polyamine spermidine may have promoted demixing of the lipid components and the formation of domains that are leaky at the discontinuities present at domain boundaries. Amphiphilic molecules like arachidonic acid, LPA, LPC, diacylglycerol, and monoacylglycerol may have promoted disorder in the lipid bilayer. The peptides A-β and amylin may have formed peptidic channels in the bilayer. Osmotic shock may have promoted transient rupture of the bilayer due to mechanical stress.

Table 4. Summary of the Effects of Annexins A5 and A6 on the Rate of Release of CF from Liposomes Subjected to Stresses[a]

agent or action	annexin A5	annexin A6
none (baseline)	protection	protection
arachidonate	protection	disruption
lysophosphatidic acid (LPA)	protection	disruption
lysophosphatidylcholine (LPC)	protection	protection
diacylglycerol	protection	disruption
monoacylglycerol	protection	disruption
spermidine	protection	protection
amyloid-β	protection	protection or disruption[b]
amylin	protection	protection
osmotic shock	protection	protection

[a]The annexins either provided protection (reduced the rate of release of CF) or caused disruption (increased the rate of release of CF). [b]Annexin A6 either provided protection or caused disruption in the presence of amyloid-β depending on the concentration of the annexin.

Just as the molecular details of the disruption events are not well-defined, the mechanism of protection by the annexins may vary in the case of different challenges. It is well-known that the annexins bind to the phospholipid headgroups, and this may have resulted in a rigidification of the bilayer,[52,53] preventing the entry of lytic agents, or may have stabilized the bilayer after the entry of such agents. The annexins may also have prevented the demixing of lipids and the formation of leaky domain boundaries. The concentrations of annexin A5 found to be near maximally effective in providing membrane protection were sufficient to provide near-complete coverage of the membrane surface. The highest annexin A5 concentration usually screened was 4.8 μg/mL or 0.14 μM. The phospholipid concentration (PS, PC, and PE combined) was approximately 31.8 μM. Of this, only half, or 15.9 μM, would be accessible on the outer leaflet of the vesicle bilayer. Therefore, the system contained a ratio of one annexin molecule to 116 exposed phospholipids. Detailed analyses of the binding of annexin A4 or A5 to phospholipid bilayers by several techniques suggest the maximal binding occurs with a footprint of the annexin covering 30−60 phospholipids.[54−57] Therefore, the area of lipid protected by the annexin appears to extend somewhat beyond the physical footprint of the protein on the membrane. The annexin may have caused the enrichment of the negatively charged PS at the binding site[11] and thus depleted regions of the membrane beyond the annexin footprint of PS. If a lytic agent, such as spermidine or A-β,[58] needed to interact with PS to initiate damage, then this depletion of PS could have extended the area of protection beyond the annexin footprint. Annexin A6 was found to be protective at a similar mass concentration (4−5 μg/mL) that would represent half the molar concentration of protein molecules relative to annexin A5, but a similar molar concentration of the annexin core domains because each annexin A6 molecule contains two core domains.

Although in some cases the annexins may have prevented disruptive agents from interacting with the bilayer and exerted a protective effect through this mechanism, it is notable that the effects of the annexins on stabilizing the bilayer were generally similar if the annexins were added before or after the lytic agents. Therefore, although the annexins may be able to interfere with the initial interaction of the agents with the bilayer, they also are protective after the agents are added and have initiated leakage pathways.

Protection from Membrane-Disrupting Peptides. Annexin A5 has previously been reported to protect liposomes with simple compositions (PS and PC) from interaction with A-β[58] or amylin.[59] Protection from A-β was suggested to be due to competition between A-β and annexin A5 for PS as a site of initial interaction on the membrane.[58] Protection from amylin was suggested to be due to binding of the annexin to soluble oligomers of amylin before they were able to attack the membrane.[59] However, again, the ability of the annexins to protect the membrane after leakage due to the peptides has already been initiated, as shown here, suggests a different mode of protection or repair may also occur.

In some cases, permeabilizing peptides are thought to act in a graded fashion in that they introduce a leak in individual vesicles that continues over time,[60,61] while in other cases the peptides may cause an "all-or-none" release from individual vesicles in a population over time.[60,62] All-or-none leakage from

individual vesicles in a population over time could have an appearance in the type of kinetic analysis of CF release performed in this study similar to that of a slow release from all of the vesicles in the population. In the case of a continuous release from all the vesicles, the action of the annexin to stop this leak could be viewed as "repairing" the leak in the vesicles. In the case of all-or-none release, the lytic agent may be first bound to the vesicle surface but does not cause a breakdown until it enters or disrupts the bilayer in a stochastic process. In this case, the annexin could be viewed as "protecting" the membrane by preventing this secondary process of membrane disruption from occurring. Either type of action of the annexin could be biologically important. Direct repair of a leaky membrane would obviously be advantageous. However, if membrane-damaging agents first cause a small leak of calcium, then annexins could presumably bind to the membrane at the site of calcium entry and prevent further damage to the membrane as the lytic agent continues to accumulate and act on the membrane.

Protection from Osmotic Shock. The ability of the annexins to protect membranes against osmotic shock might also occur through more than one mechanism. The annexin could strengthen the membrane, accelerate resealing of the membrane after breakage, or both. In one of the procedures used here, the annexin was initially bound to the vesicle in the presence of calcium, but then the osmotic shock was induced by reducing osmotic support while simultaneously removing calcium (Figure 12B). In this case, it is likely the most important role of the annexin in protecting the membrane was to make the membrane more difficult to rupture, although a promotion of resealing of the membrane might also have occurred if the off rate of the annexin in the absence of calcium was slow relative to the rate of membrane resealing.

Possible Differences between the in Vitro Model and Mechanisms in Vivo. Some of the amphiphilic agents tested here such as arachidonate and LPA are likely to be generated in situ in cells, i.e., within a membrane, and not added externally as was done here. Such agents may also not occur in vivo at concentrations relative to the total lipid concentration as high as those used here. However, such amphiphiles are likely generated locally by enzymatic means and therefore may transiently exist at local concentrations that are similar to the relative concentrations used here. Although experimental measurements of the concentrations in cells of many of the lytic agents studied here are very sparse in the literature, some data are available for the amphiphile arachidonic acid and the polyamine spermidine. Liberation of free arachidonic acid from phospholipids during the activation of blood platelets has been studied extensively.[63] Taking literature values for total arachidonic acid of 1.6−2.3 $\mu g/10^8$ platelets[64] and of total lipids (phospholipids and cholesterol) of 42.3 $\mu g/10^8$ platelets[65] and the observation that 80% of the total arachidonic acid can be liberated by the action of phospholipase during platelet activation,[64] one can calculate that the level of free arachidonic acid in activated platelets could be as high as 3−4% of total lipid on a mass basis. In experiments reported here with arachidonic acid and liposomes (e.g., Figure 5B), the ratio of arachidonate to total liposome lipids was 8%. As the arachidonate is cleaved from phospholipids, the amounts of lysolipids in cells might be similar on a transient basis. However, there are important caveats associated with making these comparisons. In the platelet, it is not likely the arachidonate would be uniformly distributed in all of the platelet membranes, leading to an underestimate of local arachidonate concentrations. On the other hand, the metabolism of the arachidonate by oxidative enzymes could significantly lower the concentration.

In the case of the polyamines, spermidine, spermine, and putrescine, these compounds are known to be present in cells at concentrations as high as low millimolar. In a survey of mammalian tissues, the spermidine content was found to range from 0.13 to 8.6 μmol/g wet weight of tissue.[66,67] From this, it can be inferred that spermidine would be present in the low millimolar range if it were free in solution. However, these are average values, and the distribution of spermidine in cells is poorly understood. Spermidine was used in the experiments described here at a concentration of 2 mM, comparable to the average bulk concentrations of spermidine in tissues.

The annexins were added to the same side of the membrane in these experiments as the lytic agents. In vivo, the geometry may be reversed in some cases. A-β peptides, for example, would be expected to attack membranes from the extracellular side, while the annexins would be present on the intracellular side. It is unclear if the annexins could provide protection from a position on the other side of the membrane. However, to the extent that the permeation pathway for CF must go all the way through the membrane, it is plausible the annexins would be protective in such a case as well by stabilizing bilayer structure while binding to the opposite side of the membrane. EPR studies of spin-labeled phospholipids have demonstrated that binding of annexin A5 to the phospholipid headgroups causes rigidification the hydrophobic bilayer interior at least to a depth of the C-12 position of the fatty acyl chains.[52] The binding of annexin A2 has also been reported to reduce the fluidity of the bilayer interior as detected with fluorescence probes.[53] Additional experimental approaches will be needed to fully assess the stabilizing effects of the annexins in a transbilayer geometry, such as the use of supported planar bilayers where the annexins may be applied on the opposite side of the membrane from the lytic agent.[18]

Although the annexins had protective effects after they were "settled" on the membrane, the initial binding interaction did in some cases lead to a transient breakdown in the permeability barrier. This effect was strengthened by the presence of some of the lytic agents, e.g., annexin A5 and spermidine (Figure 8B). Evidently, the initial binding of the annexin involves significant rearrangements of the bilayer structure that then are quickly resolved restoring the permeability barrier. In vivo, such a transient breakdown of the membrane might constitute a significant initial cost associated with the subsequent establishment of a more secure membrane barrier by the annexin.

Comparisons between Annexins. Although the model system used here has its limitations, the important message seems to be that the annexins can be protective for membranes exposed to a wide variety of permeating stresses. There were, however, some striking differences between the behavior of annexins A5 and A6 in certain cases. Most significantly, annexin A6 was deleterious and exacerbated damage caused by some agents under some conditions: arachidonate, LPA, diacylglycerol, monoacylglycerol, and A-β at some annexin concentrations. This might have been due to different headgroup specificities of the annexin core domains in annexin A5 versus annexin A6. However, it may also have had to do with a different type of interaction with the membrane of the linked core domains of annexin A6 compared to that of the single core domain of annexin A5 as this linkage is flexible and may even allow the

core domains of a single annexin A6 molecule to interact with the membrane with opposite faces.[68] Indeed, our initial preliminary experiments with a preparation of annexin A6 suggested this annexin is highly protective of membranes challenged by LPA and diacylglycerol. However, it was subsequently found that the preparation of annexin A6 used for these preliminary experiments had been cleaved between the two core domains during prolonged storage, as indicated by sodium dodecyl sulfate gel analysis. Uncleaved preparations had the deleterious effects described in Results. Evidently, one or both of the core domains of annexin A6 can in isolation exhibit protective effects more generally similar to those seen with annexin A5.

Interestingly, preliminary experiments with human annexin A1 demonstrated that this protein was also protective against baseline leakage and damage due to LPA. Because this annexin caused extensive aggregation of the liposomes that may have also contributed to protection of the membrane, these studies were not pursued in detail. Nonetheless, it was apparent in these preliminary studies that the kinetics of protection of the membrane after the addition of annexin A1, which occurred in <1 min, were similar to those seen with annexin A5 and much more rapid than the kinetics of extensive membrane aggregation induced by annexin A1 that occurs over several minutes. Therefore, it appears that the ability to protect membranes in this manner might be a general property of the annexin core domains.

Significance. One may speculate that the ability of annexins to protect the permeability barrier of cell membranes may have been of paramount evolutionary importance, explaining the high degree of conservation of the basic properties of the annexin core domain. Once developed for this primordial function, the domain may have been adopted to mediate additional calcium-regulated functions on the membrane such as organization of lipid domains, recruiting other proteins to the membrane, promoting membrane interactions in membrane trafficking, and a host of other intracellular and extracellular functions that have been identified or proposed for annexins.[4,69]

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Funding

This study was supported by Award SC090495 (Contract W81XWH-10-1-0777) from the U.S. Department of Defense Spinal Cord Injury Research Program of the Office of the Congressionally Directed Medical Research Programs.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

A-β, human amyloid-β peptide residues 1−42; assay buffer, 100 mM KCl, 50 mM HEPES-NaOH (pH 7.4), and 1 mM $CaCl_2$; CF, 5,6-carboxyfluorescein; cps, photomultiplier counts per second; FCCP, carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone; LPA, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphate; LPC, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; PC, 1,2-dioleyl-*sn*-glycero-3-phosphocholine; PE, 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine; PS, L-α-phosphatidylserine from porcine brain.

ADDITIONAL NOTE

[a]Portions of the data included here were presented in preliminary form at the 6th International Conference on Annexins in Barcelona, Spain, in August 2011, at the 56th Annual Meeting of the Biophysical Society in San Diego, CA, in February 2012, and at the 28th Annual Katz Symposium in the Department of Biology, University of Virginia, in May 2012.

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